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A QUANTITATIVE ANALYSIS OF LECTIN-MEDIATED AGGLUTINATION OF CELLS FROM DIFFERENT REGIONS OF THE GASTRULATING CHICK EMBRYO. EFFECT OF LECTINS ON ADHESION AND MORPHOGENESIS OF CELL AGGREGATES

by

© J. ROBERT PHILLIPS

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE
IN
CELL BIOLOGY

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ABSTRACT

The lectin-mediated agglutinability of cells dissociated from gastrulating chick embryos, and the effect of lectins on cell aggregate morphogenesis were investigated. In order to measure subtle differences in agglutinability the agglutination response was quantified by using the Coulter Counter.

Cells dissociated from the area pellucida and those obtained from the endoderm of the area opaca are agglutinated by Concanavalin A (Con A), wheat germ agglutinin (WGA), and *Ricinus communis* agglutinin (RCA). The greatest agglutination response is obtained with the latter lectin. A pretreatment with neuraminidase renders the cells agglutinable with soybean agglutinin (SBA). Cells from the area pellucida are more agglutinable with Con A than are area opaca endoderm cells. This difference possibly reflects tissue specific differences in the availability or display of surface glycoproteins acting as Con A receptors on these cells.

Trypsinization of area opaca endoderm cells enhances their agglutinability with Con A, WGA, and RCA. Area pellucida cells dissociated with trypsin have an enhanced SBA-mediated agglutinability compared to those dissociated with EDTA. This trypsin-mediated increase of agglutinability may be due to removal of protease sensitive materials at the cell surface and/or a trypsin-induced increase in the mobility of cell surface receptors.

Cells dissociated from whole blastoderms reaggregate in culture and undergo cell sorting and histotypic differentiation. The presence of WGA, Con A, or succinylated Con A has little effect on this

developmental pattern. RCA, however, has a profound effect, inhibiting both cell adhesion and the cell sorting in the aggregates.

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LIST OF ABBREVIATIONS

AP	Area pellucida
AOEn	Area opaca endoderm
BSA	Bovine serum albumin
CMF PCS	Calcium, magnesium-free Pannett and Compton's saline
Con A	Concanavalin A
DNase	Deoxyribonuclease
EDTA	Ethylenedinitrilotetraacetate
FCS	Fetal calf serum
GalNac	N-acetyl-D-galactosamine
GlcNac	N-acetyl-D-glucosamine
NaNase	Neuraminidase
PCS	Pannett and Compton's saline
RCA	*Ricinus communis* agglutinin
SBA	Soybean agglutinin
SBTI	Soybean trypsin inhibitor
TDG	Thiodigalactoside
WGA	Wheat germ agglutinin

INTRODUCTION

In 1907 Wilson first demonstrated that cells were capable of exercising selectivity in their interactions with other cells. Wilson mechanically dissociated living sponges into cell suspensions by forcing them through bolting cloth immersed in sea water. The cells obtained in this manner were observed to reaggregate, and in some cases, these aggregates developed into functional sponges. When cells from two different species of sponge, distinguishable by their distinctive coloration, were combined, Wilson observed that the aggregates formed were composed entirely of cells from one or the other species. His experiments suggested that the dissociated cells possessed some mechanism of discrimination which enabled them to preferentially adhere to homospecific cells.

Holtfreter (1939) subsequently demonstrated that this selectivity was also present in amphibian embryos, however, this appeared to be a tissue-specificity rather than the species-specificity observed by Wilson (1907) in the sponge system. Holtfreter cultured tissue explants from amphibian embryos at stages ranging from late blastula to neurula. He observed that the explants retained their embryological identities and interacted with other explants in a pattern that was reminiscent of their interactions *in vivo*. When explants of ectoderm and endoderm were cultured in close approximation, the ectoderm initially surrounded the endoderm. With continued culturing, however, the two cell types separated from each other. If mesoderm was also included with ectoderm and endoderm, the resulting aggregate was often organized in a pattern which was very similar to that of the embryo with ectoderm on the outside, endoderm in the center, and a layer of mesenchyme separating these two tissues. Experiments with older tissues also revealed that isolated tissue fragments possessed the necessary information to undergo reorganization and differentiation into normal embryonic structures. From these observations, Holtfreter proposed that the adhesive properties observed *in vitro* and possibly morphogenesis *in vivo* were due to what he termed tissue affinities. He suggested that these affinities were intrinsic properties of the tissues and may be either positive or negative. For example, ectoderm has a positive affinity for other ectoderm or mesoderm but a negative affinity for endoderm. These affinities may be graded in their intensity, and developmentally regulated changes in affinities may explain the migratory behavior of tissues during embryogenesis, for example, the epibolic movements of ectoderm over endoderm during gastrulation.

This theory was expanded by Townes and Holtfreter in 1955 when they postulated that affinity was an intrinsic property of single cells. They dissociated amphibian embryos into single cell suspensions by briefly subjecting the embryo to alkaline pH. When the cells were cultured at physiological pH they recombined into mixed aggregates. Within the aggregates cells sorted out according to tissue type; in some cases, the aggregates underwent a pattern of differentiation similar to that observed in vivo. For example, if epidermal and mesodermal cells were combined, the resulting aggregate consisted of mesodermal cells in the center which were surrounded by an epidermis. If cells of the medullary plate and archenteric roof were combined with epidermal cells, the aggregate formed consisted of neural cells organized as brain like tissue surrounded by an epidermis. Differentiated structures such as eye vesicles or nasal placodes were sometimes present.

In early studies on the reaggregation of dissociated cells from gastrulating chick embryos Zwilling (1960, 1963) reported that although these cells underwent limited differentiation there was no evidence of cell sorting. Zwilling (1963) concluded that cells from primitive streak stage chick embryos, which are at early stages of differentiation, do not possess the mechanism responsible for the sorting out of cells in mixed aggregates. It has been demonstrated subsequently, however, that cells from much younger chick embryos already exhibit selective cellular affinities. Cells dissociated from unincubated blastoderms and cultured under a variety of experimental conditions reassociated into aggregates in which cell sorting had occurred (Zalik and Sanders, 1974; Eyal-Giladi et al., 1975; Macarek, 1975). These aggregates characteristically consisted of an inner phase of compact cells which was surrounded by a layer of loosely adherent cells. Zalik and

Sanders (1974) suggested that the outer region was probably derived from hypoblast cells on account of its morphological similarity to yolk sac endoderm, and the inner region was formed by epiblast cells. This was confirmed by Eyal-Giladi et al. (1975) who first radiolabelled either the hypoblast or epiblast before combining its cells with cells from the other, nonlabelled, layer. Macarek (1975) reached the opposite conclusion; i.e., the compact cells were derived from the hypoblast, but his criteria for identifying the cells' origin were less stringent.

Cells from chick embryos at gastrula stages have also been cultured under reaggregating conditions and found to undergo cell sorting and differentiation into recognizable cell types such as yolk sac endoderm, blood elements, and cartilage (Sanders and Zalik, 1976). Experiments involving the aggregation of a mixed population of cells from the area opaca (Miura and Wilt, 1970) as well as pure suspensions of endodermal cells from the same area (Milos et al., 1979a) have also shown the ability of cells from young chick embryos to adhere and undergo histotypic differentiation.

As embryonic development proceeds, the cognitive capacities of cells become more refined. Differentiation of the three germ layers into tissues and organ rudiments is accompanied by a corresponding diversification of cellular affinities. The aggregative behaviour of cells dissociated from older chick embryos has been examined extensively and well documented. In 1952 Moscona introduced the use of trypsin to facilitate the dissociation of older embryonic tissues. Leg rudiments of four day embryos were dissociated with this enzyme and the cells were allowed to reaggregate. It was observed that the chondrogenic cells sorted out internally to the myogenic cells and that the former cells differentiated into cartilage (Moscona and Moscona, 1952).

In 1961 Moscona introduced a technical modification to aggregation assays in which aggregation was facilitated by culturing the cells in Erlenmeyer flasks which were incubated in a rotary shaker. The swirling motion of the fluid culture medium increased the probability of random collisions between cells. Under these conditions, if cells are mutually cohesive they will reaggregate rapidly provided the shear forces generated by the swirling motion are not too excessive. Cells which were dissociated from a variety of organ rudiments of chick embryos, such as 4 day limb bud, 7 day liver, and 7 to 19 day neural retina all reaggregated and exhibited specific cell sorting and histotypic differentiation (Moscona, 1961, 1965).

It is becoming increasingly apparent that cell surface components, notably molecules containing carbohydrate moieties, are involved in mediating numerous cellular interactions which require a degree of specificity. Processes such as intercellular communication, cell migration, cell recognition and adhesion appear to be at least partially dependent upon cell surface glycoconjugates (Cook and Stoddart, 1973; Roth, 1973; Talmadge and Burger, 1975; Edelman, 1976; Frazier and Glaser, 1979). The chemical properties of carbohydrates in biological systems make them ideal candidates for informational molecules. The number of possible permutations which can be attained by a polysaccharide containing a certain number of sugar molecules is considerably greater than that possible for a polypeptide consisting of a similar number of amino acid residues. Whereas protein molecules may exhibit differences by altering the amino acid sequence, an oligosaccharide may differ in the monosaccharide sequence, the nature and extent of branching, and in the anomeric configuration of the carbohydrate residues (Cook and Stoddart, 1973). All of these aspects may confer a molecular diversity which is biologically relevant. As oligosaccharides at the cell surface are usually linked to a protein or lipid molecule, the nature of this core molecule may also be important in determining the functional specificity of the entire glycoconjugate.

The presence of glycosubstances at the cell surface has been adequately demonstrated by a variety of experimental means such as electrokinetic studies, degradation assays, and histochemically (for reviews, see Martinez-Palomo, 1970; Winzler, 1970; Cook and Stoddart, 1973). Carbohydrates are present at the cell surface as glycoproteins, glycolipids, or glycosaminoglycans. The former two groups are usually present as integral membrane components, whereas glycosaminoglycans are generally present as components of the extracellular matrix. The focus of this investigation was primarily on the role of cell surface glycoproteins in cellular recognition and adhesion, thus, the following review will tend to be limited to the work relevant to this aspect. However, this is not to exclude glycosaminoglycans and glycolipids as possibly being involved in these events as well. In fact, glycosaminoglycans have been implicated to be involved in numerous aspects of morphogenesis such as cell migration and aggregation during cartilage formation (Toole, 1973). Bernfield and associates have suggested that glycosaminoglycans have a regulatory function in morphogenesis of salivary glands (Bernfield et al., 1973). Glycolipids also appear to be involved in cellular recognition; several of the blood group antigens have been shown to be glycolipids (Watkins, 1972).

The study of cell surface glycoproteins has been greatly facilitated by the use of specific carbohydrate binding proteins (lectins). Lectin molecules were first identified in a wide variety of plant tissues on the basis of their ability to cause agglutination of red blood cells. Interest in lectins among cell biologists has been generated because of the ability of these molecules to bind to carbohydrates with a high degree of specificity. The wide usage of these lectin molecules is demonstrated by the numerous recent review articles describing their identification, isolation, structure, carbohydrate specificites, and applications (Sharon and Lis, 1972; Lis and Sharon, 1973, 1977; Nicolson, 1974; Sharon, 1977; Brown and Hunt, 1978). As mentioned previously, proteins with lectin activity were first demonstrated by their ability to agglutinate red blood cells; by operational definition, therefore, lectins possess two or more carbohydrate binding sites. Agglutination presumably occurs as a result, in part, of the lectin molecule cross-linking carbohydrate molecules on adjacent cells. The ability of lectins to cause agglutination of a wide variety of cells other than erythrocytes, as well as their defined carbohydrate specificities has made possible their use as investigative probes of cell surface carbohydrates. The presence and relative abundance of specific carbohydrate molecules at the cell surface can be demonstrated by using lectins of known specificities.

The agglutination response is not fully understood, it seems to require more than just the physical presence of the lectin and the lectin receptor at the cell surface. The valence of the lectin is an important factor affecting the agglutination reaction. Monovalent derivatives of concanavalin A (Con A), prepared by succinylation (Gunther et al., 1973), or by treatment with chymotrypsin (Steinberg and Gepner, 1973), were considerably less effective than native Con A at agglutinating sheep erythrocytes, mouse spleen cells (Gunther et al., 1973), and embryonic chick neural retina cells (Steinberg and Gepner, 1973). Increasing the valence of soybean agglutinin by cross-linking molecules with glutaraldehyde enhanced its ability to agglutinate lymphocytes and erythrocytes by factors of 30 to 200 times (Lotan et al., 1973).

A vast number of transformed cells have been shown to be more agglutinable with lectins than their nontransformed counterparts (reviewed by Lis and Sharon, 1973; Nicolson, 1974, 1976; Brown and Hunt, 1978). The most plausible explanation for this increased agglutinability is that the nontransformed cells possess fewer lectin binding sites. However, in the majority of cases examined, this assumption has been rejected. Studies using labelled lectin have revealed that, in most cases, nontransformed cells bind as much lectin as tranformed cells (Nicolson, 1976). Exceptions to this, however, have been reported by Noonan and Burger (1973a, 1973b) who examined Con A binding to a number of normal and transformed cell lines and found that transformed cells bound more lectin. Protease treatment has also been reported to enhance lectin mediated agglutinability of a variety of cell types (Burger, 1969; Inbar and Sachs, 1969; Nicolson and Blaustein, 1972; Kleinschuster and Moscona, 1972; Zalik and Cook, 1976). This effect, also, does not appear to be due to differences in the number of lectin receptors present (Inbar et al., 1971; Nicolson, 1973a). Nicolson (1976) has stated that "there is no obvious relationship between the number of lectin receptors on a given cell and its agglutination characteristics."

Nicolson (1971, 1972) proposed that the mobility and distribution of lectin receptors in the plasma membrane may be responsible for the higher agglutinability found in transformed and protease treated cells. Nicolson provided evidence that lectin receptors on transformed and protease treated cells tended to be organized in patches or clusters, whereas, these receptors were randomly distributed on normal cells. It has been subsequently demonstrated, however, that the lectin itself is instrumental in inducing the reorientation of the receptors. If lectin was added to transformed cells which had either been prefixed, or maintained at low temperature, the receptors were present in a randomly dispersed arrangement. Warming the cells, or adding lectin before fixation, showed the receptors orientated in patches or clusters (Inbar and Sachs, 1973; Nicolson, 1973b). This does not conclusively demonstrate, however, a causal relationship between the mobility and distribution of lectin receptors and the degree of agglutination.

The cytoskeletal elements, microtubules and microfilaments, have been implicated to be involved in regulating the position of cell surface lectin receptors (Edelman, 1976). Treatments that affect microtubules and microfilaments have been shown to alter both the distribution of cell surface receptors and the lectin-mediated agglutinability. Important among these, are the experiments utilizing colchicine, which prevents polymerization of microtubule subunits (Borisy and Taylor, 1967), and cyotchalasin B, which disrupts microfilaments (Lin and Spudich, 1974; Weihing, 1976). Both drugs have been reported to alter the distribution of surface receptors as visualized with fluorescent labelled antibodies (Edelman et al., 1973; de Petris, 1974; McDonough and Lilien, 1975; Nicolson and Poste, 1976). Colchicine has increased the Con A-mediated agglutinability of mouse LM cells (Rittenhouse et al., 1974) but decreased Con A-mediated agglutination of SV 3T3 cells, polymorphonuclear leucocytes, and hepatoma cells (Yin et al., 1972; Berlin and Ukena, 1972; Nakamura and Terayama, 1975). Neither colchicine nor cytochalasin B affected lectin-induced agglutination of embryonic amphibian cells (Fraser and Zalik, 1977) or embryonic chick cells (Phillips and Zalik, 1978).

Although there is a vast amount of information on factors affecting lectin-mediated agglutination, this phenomenon appears to be very complex and is, as yet, poorly understood. It still remains to be demonstrated conclusively that agglutination actually occurs as a result of lectins forming cross-links between carbohydrate molecules on adjacent cells, although, this seems the most likely explanation. Despite the lack of information on the process agglutination assays are used extensively because they are a fast and convenient method of obtaining information about the nature of cell surface carbohydrates.

A considerable amount of our current knowledge of the molecular mechanisms mediating adhesive specificity has been obtained from studies in sponges and cellular slime molds. These systems will be discussed here as they have yielded a great deal of information, some of which appears to be paralleled by vertebrate aggregation systems.

Wilson's (1907) initial observation that dissociated sponge cells exhibit species-specificity has since been confirmed by other workers examining a variety of species (Spiegal, 1954; Humphreys, 1963; Moscona, 1968; McClay, 1971). A factor which is reported to mediate aggregation is present in supernatants of sponge cells dissociated at 5°C in calcium, magnesium-free sea water (Humphreys , 1963; Moscona, 1968). Cells which are dissociated in this manner will not aggregate in sea water at 5°C but will aggregate at temperatures ranging from 18 to 25°C. If the supernatant containing the aggregation factor is added to cells maintained at 5°C, cell aggregation occurs (Humphreys, 1963). The aggregation factor appears to be species-specific; the factor from *Microciona prolifera* had no effect on the aggregation of cells from *Haliclona occulata* at 5°C and likewise, the factor from *H. occulata* did not increase aggregation of *M. prolifera* cells (Humphreys, 1963). The factor requires the presence of calcium for activity; it is apparently lost from the cell surface during dissociation in calcium, magnesium-free sea water but it can be activated by the addition of calcium (Moscona, 1968). The activity of the factor is sensitive to heat denaturation and digestion with pronase or α-amylase (Moscona, 1968). Chemical characterization of the aggregation factor from several species of sponge has revealed that it is a high molecular weight proteoglycan (Margoliash et al., 1965; Henkart et al., 1973; Cauldwell et al., 1973). Electron microscopic observation of the factor from two species (*Microciona parthena* and *Geodia cydonium*) has revealed a fibrous structure which is organized into a "sunburst" pattern of a central circle with radiating fibres (Henkart et al., 1973; Müller et al., 1978). Molecules which can act as receptors for the aggregation factor have been isolated from sponge cells (Weinbaum and Burger, 1973; Müller et al., 1976). Various mechanisms explaining the interaction of receptor and aggregation factor in specific cell adhesion have been postulated; however, the exact nature of the interaction still remains to be established.

The cellular slime molds also provide an interesting system for the study of adhesion. In the vegetative, or growth phase, the mold exists as single cells which are aggregation imcompetent. Depletion of the food supply results in a chemotactically directed aggregation. During aggregation cells adhere to each other to form a large multicellular pseudoplasmodium which will migrate and eventually form a fruiting body (Grant, 1978).

Glycoprotein molecules appear to be important in the acquisition of the developmentally regulated adhesiveness. Current information from two laboratories seems to indicate that there are two mechanisms involved in the expression of adhesiveness. Gerisch and associates have prepared univalent antibody fragments (Fab) against surface antigens of aggregation competent *Dictyostelium discoideum*. Two Fab molecules of different specificities have been isolated which block the adhesion of *D. discoideum* cells (Beug et al., 1970, 1973). The target molecules of the Fabs, termed contact sites, are apparently involved in two aspects of adhesion. Contact site 'A' is present only on aggregation competent cells and appears to be involved in end to end adhesion; contact site 'B' is present on both growth phase and aggregation competent cells and is apparently involved in side to side adhesion (Beug et al., 1973). Contact site 'A' has been purified from the plasma membrane and shown to be a glycoprotein which binds Con A (Heusgen and Gerisch, 1975; Eitle and Gerisch, 1977; Müller et al., 1979). The contact site 'A' shows species-specificity as Fab directed against site 'A' from *D. discoideum* does not block adhesion of *Polysphondylium pallidum*; the converse situation also holds true (Bozzaro and Gerisch, 1978). The actual role of the contact sites in adhesion is still unestablished. The simplest mechanism possible is that contact sites on adjacent cells interact directly with each other, although, the experimental evidence does not exclude the possibility of multicomponent systems (Müller and Gerisch, 1978).

The work of Barondes, and others in his laboratory, has primarily been concerned with the role of endogenous lectins in the adhesive phenomena of slime mold cells. Rosen et al. (1973) demonstrated that lectin activity is present in protein extracts of aggregation competent *D. discoideum* cells. The presence of the lectin is developmentally regulated as lectin activity is not detected in extracts of vegetative phase cells. The lectin specificity is directed against carbohydrates with a galactose configuration. Purification of the active protein fraction has revealed that there are two carbohydrate-binding proteins which differ in their amino acid composition, carbohydrate specificities, and their developmental regulation; the lectins appear at different rates as cells become cohesive. These two lectins were named discoidin I and II (Simpson et al., 1974; Frazier et al., 1975). A molecule with lectin activity has also been isolated from aggregative cells of Polysphondylium pallidum (Rosen et al., 1974). The carbohydrate specificity of this "pallidin" is also directed against galactose residues, however, it differs slightly from the discoidins as the most potent inhibitor of pallidin induced hemagglutination is lactose, whereas N-acetyl-D-galactosamine and D-fucose are the most potent inhibitors of discoidin I and II respectively (Rosen et al., 1974; Frazier et al., 1975). In both species the lectins appear to be present at the cell surface. This has been demonstrated by the ability of intact cells to bind erythrocytes and by immunofluorescent and immunoferritin binding studies (Rosen et al., 1973; Chang et al., 1975, 1977).

Some evidence for a role of these lectins in adhesion has been obtained by studies showing that factors that block lectin hemagglutinating activity also cause a reduction in adhesion of aggregation competent cells. High concentrations of simple sugar haptens reduced the aggregation of P. pallidum cells (Rosen et al., 1974). Asialofetuin, a glycoprotein with terminal galactose residues, has also been shown to inhibit aggregation (Rosen et al., 1977), as have antibody fragments directed against pallidin (Rosen et al., 1976, 1977). These results are open to a certain amount of criticism as the reduction of aggregation was observed under so called "permissive" conditions in which the cells were heat treated, or cultured under hypertonic conditions or in the presence of antimetabolites. In isotonic culture media the aggregation was only minimally affected by the aforementioned treatments.

The lectin activities studied in quite a few species of slime molds exhibit slight differences in their carbohydrate specificities. It has been suggested that these differences may be involved in conferring species-specificity, although as yet, there is no experimental evidence to support this idea (Rosen et al., 1975; Barondes and Haywood, 1979).

The relationship between the lectins studied by Barondes' group and the contact sites described by Gerisch and coworkers is still unclear. It appears that there may be two chemically and functionally distinct systems. Contact sites 'A' can be purified free from discoidin; the contact site itself has no demonstratable lectin activity. Antibody fragments prepared against contact sites or against endogenous lectins appear to be directed against different antigenic components at the surface (Huesgen and Gerisch, 1975; Müller and Gerisch, 1978). It is possible, however, that continued investigation will reveal an interdependence of the two systems.

Specific cellular adhesion in vertebrates also appears to be partially dependent upon cell surface glycoproteins. Few vertebrate systems have been worked out in as great detail as the sponges and slime molds, however, information from a wide variety of sources has provided at least circumstantial evidence implicating cell surface components in cellular recognition and adhesion.

Evidence for a role of cell surface glycoproteins in adhesive phenomena has come from experiments in which these surface components were either removed or modified in some way. Neuraminidase is a glycosidic enzyme which cleaves the α-ketosidic linkage joining a terminal acylated neuraminic acid to another sugar or sugar derivative (Gottschalk, 1958). Kemp (1968, 1970) reported that neuraminidase reduced the aggregation of embryonic chick muscle cells. Increased aggregation of cells treated with neuraminidase was observed by Vicker and Edwards (1972) using BHK 21 cells and by Lloyd and Cook (1974) working with 16 C malignant rat dermal fibroblasts. Another glycosidic enzyme, β-galactosidase, has also been shown to affect adhesion. Roth et al. (1971a) demonstrated that this enzyme caused an increased frequency of nonspecific adhesions formed between embryonic chick neural retina aggregates and other heterologous tissues such as liver.

Evans and Jones (1974) demonstrated that a non-agglutinating form of Con A prepared by trypsinization of native Con A inhibited the aggregation of embryonic chick muscle cells. This inhibition was not observed if the hapten α-methyl-D-glucopyranoside was included with the Con A derivative. Evans and Jones suggested that the inhibition of aggregation was due to the Con A binding to the receptor sites and causing surface associated changes which hampered adhesion. Steinberg and Gepner (1973), however, reported that the presence of univalent Con A did not affect the aggregation or cell sorting of embryonic chick cells. Recently, Con A has been shown to inhibit the adhesion of human skin fibroblasts to a plastic substrate (Prinz and Von Figura, 1978) and the adhesion of embryonic chick neural retina aggregates to cell layers of the same type (Letourneau, 1979). In both of these studies it was surmised that Con A exerted its effect by affecting the mobility or distribution of receptors at the cell surface. Low concentrations of lectins also have been shown to disrupt the migration of primordial germ cells in the chick embryo (Lee et al., 1978). The migration of primordial germ cells from the hypoblast to the germinal ridge normally occurs with a fairly high degree of accuracy. Both Con A and wheat germ agglutinin inhibited the migration of the germ cells away from the germinal crescent area of the hypoblast; the haptens for these lectins abolished the inhibition of migration. Again, it was suggested that the presence of the lectin alters the distribution of receptors at the surface, and as a consequence of this, the specific migratory capabilities of the cells are disrupted.

Lilien and his associates have presented evidence that suggests that cell surface glycoproteins are involved in mediating specific cell adhesions of embryonic chick neural retina cells. These investigators have suggested that the adhesive mechanism is a three component system, consisting of a cell surface receptor, a ligand that binds to the receptor, and an agglutinin molecule which can bind ligands (Rutz and Lilien, 1979). Evidence for this system has come from a series of experiments. Lilien (1968) reported that supernatants of monolayer cultures of neural retina cells contained a factor (termed ligand by Rutz and Lilien, 1979) that enhanced neural retina cell aggregation, but not liver cell aggregation. Supernatants of neural retina tissue culture media also contained a factor which specifically bound to neural retina cells, suggesting the presence of a cell surface receptor for the ligand (Balsamo and Lilien, 1974a). Balsamo and Lilien (1974b) also demonstrated that a third component (termed agglutinin by Rutz and Lilien, 1979) was produced by neural retina cells in culture. This agglutinin was shown to be necessary for the enhancement of aggregation induced by the ligand. The agglutinin activity was sensitive to protease treatment (Rutz and Lilien, 1979), whereas, the specific binding of the ligand to the cell surface was sensitive to glycosidase treatment (Balsamo and Lilien, 1975). According to Rutz and Lilien (1979), the simplest explanation to account for these data is that ligand molecules specifically bind to the cell surface receptors. Adhesion of cells is accomplished by the agglutinin which can cross-link ligand molecules on adjacent cells.

Moscona and coworkers also have been examining the mechanism of adhesion of neural retina cells. Recent work from Moscona's laboratory has concentrated on the isolation and chemical characterization of a molecule which specifically enhances aggregation of neural retina cells. Hausman and Moscona (1975) purified an adhesion promoting factor from cell culture supernatants and found it to be a glycoprotein with a molecular weight of 50,000. Its activity as a specific aggregation promoting factor required integrity of the protein portion but not the carbohydrate portion. This molecule was referred to as "cognin" by Moscona et al. (1975). Cognin has been purified from isolated plasma membranes of neural retina cells which indicates a possible cell surface localization (Hausman and Moscona, 1976). Studies involving immunolabelling techniques have provided additional evidence for a cell surface location of this molecule (Ben-Shaul et al., 1979). The presence of cognin at the cell surface appears to be related to the adhesive state of the cells. Cognin did not appear to be present on the surfaces of freshly trypsinized cells, which do not aggregate; but after a recovery period in which adhesiveness is regained, the presence of cognin could be demonstrated. Also, cells from older neural retinas, which are not very adhesive, apparently have less cognin that younger cells (Hausman and Moscona, 1979; Ben-Shaul et al., 1979).

Rutz and Lilien (1979) have suggested that cognin is possibly the same molecule as the agglutinin which they have described. This suggestion is based on several similarities between the molecules, such as the protease sensitivities, the specificities, and the time and temperature profiles of activity. Clarification of this hypothesis, however, awaits the isolation and characterization of the agglutinin.

Roseman (1970) and Roth et al. (1971b) proposed a model for intercellular adhesion which involves enzyme-substrate interactions at the cell surface. It was suggested that complexes between cell surface glycosyltransferases and cell surface glycoproteins may provide a means of establishing specific intercellular adhesions. The glycosyltransferases are enzymes which catalyze the transfer of a monosaccharide residue from a sugar nucleotide to the nonreducing terminus of a specific sugar acceptor. The presence of these enzymes at the surface of a wide variety of cell types has been reported by numerous investigators (reviewed by Shur and Roth, 1975). The model proposed by Roseman (1970) has the possibility of being a highly regulated process by controlling such factors as the presence of a particular enzyme at the cell surface, the enzyme activity, the presence of the proper substrate and the availability of sugar nucleotide donors. It also allows for a process, termed intercellular modification by Roseman, whereby the glycosyltransferase on one cell can catalyze the addition of a sugar residue to a glycoprotein chain on an adjacent cell. As the modified glycoprotein is no longer a substrate for the enzyme, the complex dissociates. If an enzyme specific for the terminal carbohydrate of the modified glycoprotein is not present, the final result may be the dissociation of the cells. If the enzyme specific for the modified glycoprotein is present, but the sugar nucleotide donor is not, the final result may be an increased adhesion. This model may, thus, provide an explanation for the observed changes in cellular specificity and adhesion during embryonic development.

An exciting development in the study of the molecular basis of cellular recognition and adhesion was the discovery of endogenous lectin molecules in vertebrate tissue. As cells have been demonstrated to possess lectin receptors on their surfaces, the presence of lectin molecules at the surface provides an ideal complementary molecule for recognition and binding interactions.

In an elegant series of experiments, Ashwell and his colleagues demonstrated the presence of a lectin in mammalian liver and identified its role in the regulation of circulating level of serum glycoproteins. The elucidation of this system, which has been excellently reviewed by Ashwell and Morell (1974, 1977), stemmed from the initial observations that the nature of the terminal carbohydrates on the serum glycoprotein ceruloplasmin was important for the glycoprotein to remain in circulation. If the terminal sialic acid residues were removed, thereby exposing galactose residues, the half life of this molecule in the serum dropped from a few days to a few minutes. If the galactose residues were also removed, the half life of the glycoprotein in the circulation was reextended. Adding sialic acid residues back onto the asialo-glycoprotein also extended its half life in the serum. The cleared glycoproteins (those with exposed galactose residues) were observed to collect in the parenchymal cells of the liver where they were degraded by lysosomal activity. Examination of the mechanism of binding and uptake revealed that neuraminidase treatment of the hepatic cells reduced their ability to remove asialo-glycoproteins from serum. This suggested the involvement of a surface glycoprotein on the parenchymal cells. The hepatic binding protein was isolated and in 1974 Stockert et al. reported on the ability of this molecule to agglutinate red blood cells, thus, they described it as the first lectin of mammalian origin.

The work of Ashwell and his coworkers demonstrated the first conclusive evidence of the involvement of lectin-like molecules in biological recognition phenomena. Although lectins have now been shown to be present in a wide variety of vertebrate tissues, their role is still speculative. Cell-cell interactions appear to be very complex processes and it has proven difficult to provide conclusive evidence for the involvement of lectins in this regard.

Gartner and Podleski (1975, 1976) have demonstrated that a lectin which is blocked by thiodigalactoside is present at the surface of cultured cells from rat skeletal muscle. These authors suggested that this lectin may be involved in the fusion of myoblasts, as the presence of thiodigalactoside prevented the formation of myotubes. A lectin with similar carbohydrate specificity has been shown to be present in embryonic chick muscle (Nowak et al., 1976). The activity of this lectin is developmentally regulated; at early stages of development it was present in low levels but activity rose as differentiation proceeded. Nowak et al. (1977) have purified the muscle lectin and have demonstrated its presence both within the cell and at the surface by immunofluorescent labelling techniques. The involvement of the chick muscle lectin in myoblast fusion has been questioned by Den et al. (1976), since they could not prevent myoblast fusion by the addition of thiodigalactoside.

Recently, a carbohydrate-binding component, presumed to be present at the surface of teratocarcinoma stem cells, has been implicated in cellular adhesion (Grabel et al., 1979). The carbohydrate specificity of this component is directed against oligomannosyl residues. Mannose rich glycoconjugates such as invertase and yeast mannan were the most effective inhibitors of erythrocyte binding to the stem cells. Invertase inhibited stem cell aggregation and also disrupted preformed cell aggregates.

Developmentally regulated lectin activity has been demonstrated in a wide variety of embryonic chick tissues, including brain, heart, and liver (Kobiler and Barondes, 1977; Kobiler et al., 1978). These lectins all apparently have specificities directed against β-D-galactosyl groups; thiodigalactoside and lactose were the most potent inhibitors of their hemagglutinating activity. Recently, a lectin also specific for β-D-galactosyl groups has been demonstrated in extracts from chick embryos at pregastrula and gastrula stages (Cook et al., 1979). The fact that some concentrations of thiodigalactoside inhibited the aggregation of extraembryonic endoderm cells suggests that this lectin may be involved in intercellular adhesion.

The similar carbohydrate specificies of the lectins isolated from different embryonic chick tissue does not really favour the hypothesis that these molecules are involved in mediating specific cellular interactions. However, there are possibly other factors involved. The developmental regulation of the presence of these lectins may provide some means of specificity. Also worthy of consideration is that the availability of the different receptors for specific lectins may be under some form of control. Studies on the *in situ* localization of lectin binding sites on cell surfaces of early chick embryos have revealed that some of the receptors are not homogeneously distributed. Hook and Sanders (1977) demonstrated that in pregastrula embryos all cell surfaces which were accessible to lectin, showed the presence of Con A binding sites. At the gastrula stage, however, Con A binding sites were mainly evident on the dorsal surface of the epiblast. The ventral surface of the epiblast, and the dorsal and ventral surfaces of the endoblast appeared to be relatively free of Con A binding sites. In a similar study, Sanders and Anderson (1979) examined the distribution of wheat germ agglutinin (WGA) binding sites. Again, in this study, lectin binding sites were evident on all cell surfaces of the pregastrula stage embryo. At the gastrula stage, however, the WGA binding sites appeared to be limited to the surfaces of the cells lining the cavity between the epiblast and the endoblast. Cells present within the primitive streak did not bind WGA but as the mesenchyme cells moved away from the streak they regained WGA binding sites.

That some lectin receptors are developmentally regulated was demonstrated by Kleinschuster and Moscona (1972). These investigators reported that as development proceeds, embryonic neural retina cells become less agglutinable with Con A. Trypsinization of the cells, however, increased their agglutinability with Con A. WGA did not agglutinate neural retina cells from 8 to 20 day embryos unless they were prepared by trypsinization. Zalik and Cook (1976) have also shown that liver cells from 10 to 12 day embryos were not agglutinated by WGA unless they were trypsinized. Cells from very young embryos (gastrula stage), however, were agglutinable with WGA without trypsinization.

All of the aforementioned evidence seems to indicate that changes in the cell surface glycoconjugates occur during development. The present study was undertaken to examine, in more detail, the lectin-mediated agglutinability of two cell populations dissociated from gastrulating chick embryos, and to investigate a possible role of cell surface lectin receptors in recognition and adhesion. Early embryonic cells provide an interesting system for the study of these phenomena because of the dynamic nature of the intercellular interactions which are occurring. During gastrulation in the chick, morphogenetic movements are occurring to transform the two layered embryo into a three layered one. During this process cells are breaking contact with their surrounding cells, moving through the embryo, forming new associations with other cells, and acquiring diverse differentiative pathways. Presumably, these changes in cellular affinities are expressed at the cell surface. A study of cell surface lectin receptors at this stage of development could provide meaningful information as to their role in these processes.

Due to the time spent in transport down the genital tract of the hen, the stage of development of the unincubated blastoderm can be quite variable (Eyal-Giladi and Kochav, 1976). Generally, at the time of laying, the blastoderm consists of a disc of cells (the epiblast) which is divided into two regions; the central area pellucida which will give rise to the embryo, and the peripheral area opaca which will be involved in the formation of some of the extraembryonic membranes. The hypoblast, which forms ventral to the epiblast, may be in varying stages of completion at the time of laying. The exact mechanism of hypoblast formation is uncertain, but it appears to be a combination of delamination of cells from the epiblast and an anterior migration of cells from a localized thickening in the posterior region of the blastoderm (Spratt and Haas, 1960; Vakaet, 1962, 1970; Eyal-Giladi and Kochav, 1976). The hypoblast appears to be destined to form the endoderm of the yolk sac (Rosenquist, 1971; Fontaine and Le Douarin, 1977).

At the definitive primitive streak stage of development the embryo consists of several more cell types than those present in the unincubated blastoderm. The area pellucida now consists of three layers; the dorsal epiblast, the ventral endoblast, and an intervening layer of mesoderm. The latter two layers are formed by cells invaginating through the primitive streak. The endoblast, which will become the embryonic endoderm infiltrates into the hypoblast, pushing it out towards the area opaca (Vakaet, 1970;

Rosenquist, 1972; Fontaine and Le Douarin, 1977). The area opaca consists of the dorsal epiblast, the ventral hypoblast, or endoderm, and some mesoderm in the posterior region of the area opaca (Bellairs, 1963).

Previous studies on lectin-mediated agglutination of cells dissociated from early chick embryos were performed on mixed cell suspensions from whole blastoderms (Zalik and Cook, 1976; Phillips and Zalik, 1978). The question arose as to whether cells from different regions of the embryo differed in their lectin-mediated agglutinability. It was realized that the visual assessment of agglutination utilized in the previous studies was neither sensitive nor objective enough to detect small differences in agglutinability (Phillips and Zalik, 1978). In this investigation, the lectin-mediated agglutinability of cells dissociated from the area pellucida was compared to the agglutinability of endodermal cells from the area opaca of primitive streak stage chick embryos. In order to accurately detect differences which may reflect tissue specificites it was necessary to quantitate the agglutination response. To accomplish this, an electronic particle counter was utilized to monitor the agglutionation reaction.

The lectins employed in this study, and their respective sugar specificites were: concanavalin A (Con A), α-methyl-D-mannopyranose, α-D-glucopyranose, α-D-fructofuranose, and their glycosides; wheat germ agglutinin (WGA), N-acetyl-D-glucosamine (GlcNac), and oligosaccharides of GlcNac, and N-acetylneuraminc acid;

Ricinus communis agglutinin (RCA), β-linked dissaccharides with D-galactose at the nonreducing end; and soybean agglutinin (SBA), N-acetyl-D-galactosamine (GalNac), disaccharides with GalNac at the nonreducing end, and D-galactose (Lis and Sharon, 1973).

In early chick embryos, different cell regions may be characterized by their differential cell adhesiveness. Eyal-Giladi et al. (1975) reported that the hypoblast of unincubated blastoderms could be dissociated after a much shorter exposure to trypsin than was required for dissociation of the epiblast. Milos et al. (1979a) were able to make use of the differential dissociability of the extraembryonic endoderm and the extraembryonic epiblast in order to prepare suspensions of endodermal cells. In this investigation it was found that dissociation of the area pellucida required relatively severe techniques utilizing EDTA or trypsin, whereas, the area opaca endoderm could be dissociated in a calcium, magnesium free saline.

The method of dissociation has been shown to affect the lectin-mediated agglutinability of cells. Kleinschuster and Moscona (1972) reported that trypsin dissociated cells were more agglutinable with Con A and WGA than cells dissociated by EGTA treatment. Rottman et al. (1974) also have demonstrated an enhanced Con A-mediated agglutinability of cells dissociated in trypsin compared to cells dissociated in EDTA. In the present study, the effect of the dissociation technique, and in particular trypsin treatment, on lectin-mediated agglutination was examined. The quantitative assessment of agglutination made the results amenable to statistical analysis, and as a consequence, small differences in agglutinability could be detected.

The final aspect of this investigation was an attempt to determine if cell surface lectin receptors are in some way involved in specific cell interactions. For this purpose, cells were allowed to reaggregate in the presence of low concentrations of different lectins and the effect of this treatment on cell adhesion and cell sorting was determined.

MATERIALS AND METHODS

A. Preparation of Solutions

1. Modified Pannett and Compton's Saline (PCS)

A 10 x concentrate was prepared as follows:

48.4 g	NaCl
6.2 g	KCl
3.1 g	$CaCl_2$
5.1 g	$MgCl_2 \cdot 6H_2O$
35.7 g	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES)

Distilled water to 1000 ml

pH adjusted to 7.5 with concentrated NaOH

This solution was stored at 5°C and diluted ten times with distilled water before use. This is a modification of the saline originally described by Pannett and Compton (1924) in that the phosphate buffer has been replaced with 15 mM HEPES. A calcium and magnesium-free PCS (CMF PCS) was prepared also.

2. Leibovitz (L-15) medium

Leibovitz tissue culture medium (Gibco), in powdered form, was dissolved in the specified amount of distilled water and adjusted to pH 7.5 with 1 N NaOH. The solution was sterilized by membrane filtration through a 0.22 µm Millipore filter and stored at 5°C. Special formula (#79-5107) L-15 medium without galactose and glutamine was prepared similarily and supplemented with 300 mg/l L-glutamine and 900 mg/l glucose.

3. Lectins

Concanavalin A (3 x crystallized) was purchased from Miles Biochemicals as a lyophilized powder and stored at -20°C. Con A was dissolved in PCS to obtain a solution of the appropriate concentration. This solution was always prepared shortly before its use because of the tendency of Con A molecules to aggregate in solutions above pH 7 (McKenzie et al., 1972). The activity of Con A solutions, as assessed by agglutination assays, decreased markedly after freezing and thawing.

Wheat germ agglutinin (Miles Biochemicals) was obtained as an aqueous solution containing 1.35 mg protein/ml. This solution was diluted to 1.0 mg/ml with distilled water and stored at -20°C. Further dilutions of this stock solution were made with PCS; these dilute solutions could be repeatedly frozen and thawed without any appreciable loss of activity.

Ricinus communis agglutinin and soybean agglutinin were a gift from Dr. G. M. W. Cook of the Department of Pharmacology, University of Cambridge, Cambridge, U.K. These were both provided as lyophilized powders and stored at -20°C. Dilute solutions of both lectins were prepared by dissolving the appropriate weight in PCS. These solutions retained their activity after freezing and thawing over a time period of greater than two weeks. The larger molecular weight species (120,000) of RCA was used exclusively in this investigation.

Succinylated Con A was prepared by the method of Gunther et al. (1973). A solution of Con A was prepared by dissolving 25 mg Con A in 7 ml saturated sodium acetate at room temperature. The solution was centrifuged at 900 x g (setting #7 in an International Equipment Co. clinical centrifuge) to remove any precipitate. The supernatant was transferred to a 25 ml flask containing 8 mg succinic anhydride and mixed for 90 minutes in an ice bath. The solution was dialyzed over night against distilled water at 5^{o}C (Spectrapor membrane tubing #1, M. W. cutoff: 6,000-8,000). Following dialysis the volume of the solution was reduced by approximately 30% by concentration against polyethylene glycol (M. W. 20,000). The solution was lyophilized and the product was subjected to a second derivitization by dissolving it in 5 ml saturated sodium acetate. After centrifugation at 900 x g for 15 minutes the supernatant was added to a 10 ml flask containing 8 mg succinic anhydride and stirred for 90 minutes at room temperature. After exhaustive dialysis against distilled water at 5^{o}C, the solution was lyophilized. The resulting product was stored at -20^{o}C. Dilutions at the appropriate concentrations were prepared by dissolving the succinylated Con A in PCS; this solution was used within four hours after dilution.

4. Sugar solutions (Haptens)

The haptens utilized in this study were N-acetyl-D-glucosamine

(GlcNac), N-acetyl-D-galactosamine (GalNac), β-lactose, α-methyl-D-mannoside and thiodigalactoside (TDG). These were all purchased from Sigma. The sugars were dissolved in PCS to the appropriate concentration within four hours of their use.

5. Enzyme solutions

Deoxyribonuclease I (DNase), from bovine pancreas, (Sigma, DN 100) was dissolved in PCS to give a stock solution containing 5,000 Kunitz units/ml. Aliquots of this solution were added to the cell suspensions to give a final concentration of 50 or 100 units/ml. Due to variations in the activity of different lots of this enzyme, the final concentrations ranged from 25 to 80 μg/ml.

Trypsin (Sigma, type III), from bovine pancreas, was dissolved immediately before use in CMF PCS to the appropriate concentration. The particular preparation of trypsin utilized was stated by the supplier to contain 12,000 BAEE units/mg protein.

Soybean trypsin inhibitor (SBTI), (Sigma, type 1-S) was dissolved in CMF PCS. According to the supplier, 1 mg SBTI will inhibit 1.25 mg trypsin with an approximate activity of 10,000 BAEE units/mg. In this investigation, SBTI was used at a 1:1 ratio to trypsin.

Neuraminidase (NaNase), from *Vibrio comma*, was purchased from Behringwerke. The enzyme was supplied in a sodium acetate buffer with an activity of 500 units/ml. This enzyme preparation was stated by the supplier to be free of aldolase, lecithinase C and protease.

B. Preparation of Embryos

Fertilized eggs from white Leghorn chickens were obtained from the University of Alberta Poultry Farm and incubated 18 to 20 hours at 39°C. The blastoderms were cut off the yolk and transferred to a petri dish containing PCS. Blastoderms were shaken free of the vitelline membrane and rinsed exhaustively in PCS to remove as much of the adhering yolk as possible. Embryos at the primitive streak stage, stage 4 to 5, (Hamburger and Hamilton, 1951) were transferred to L-15 medium where they remained until used. In some cases the blastoderms were stored overnight at room temperature. In this case the L-15 medium was supplemented with 20 μg/ml gentamycin sulfate (Sigma), however, in most instances embryos were used within four hours.

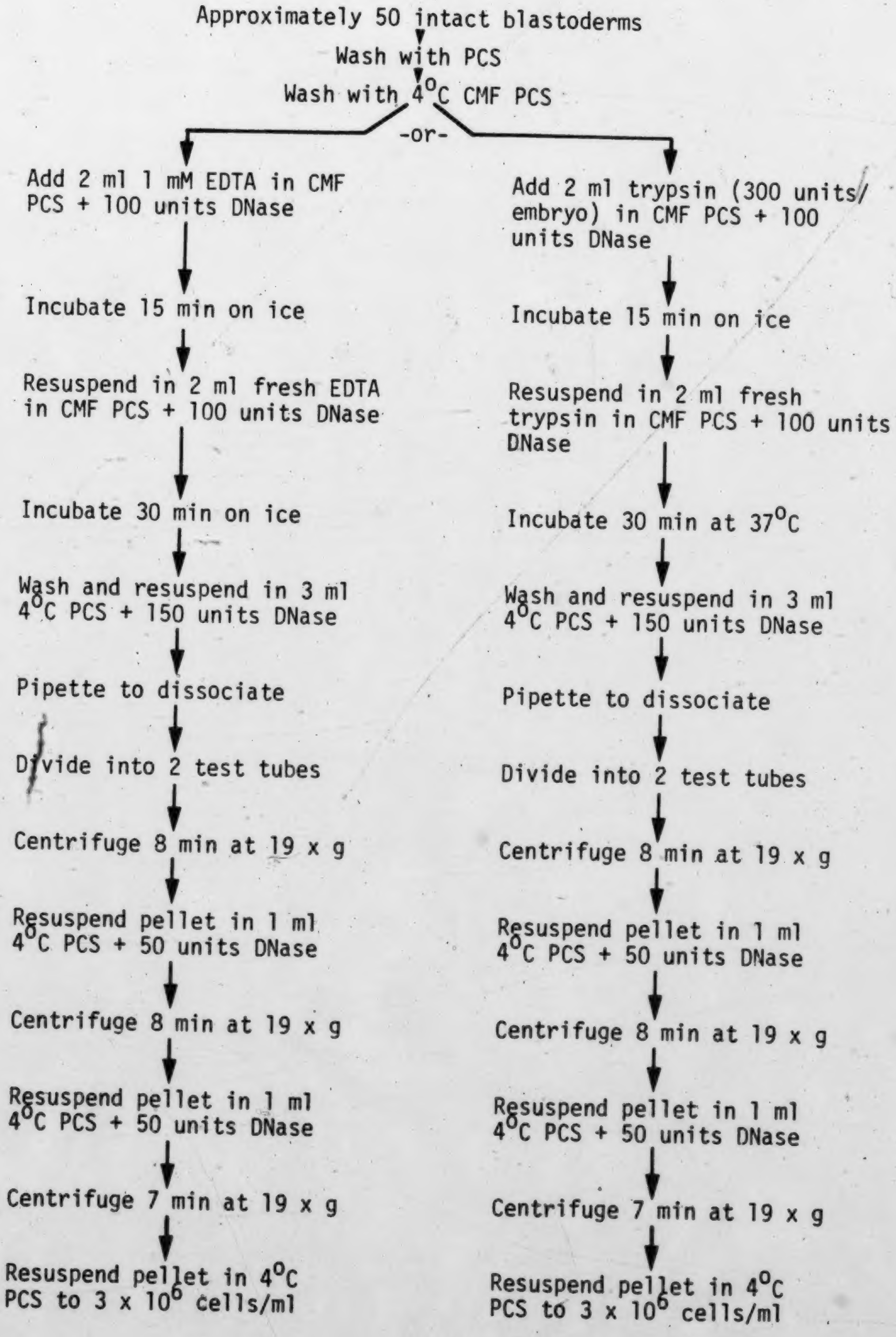
C. Preparation of Cell Suspensions

The embryos were either used intact, or dissected into two sections: the area pellucida and the area opaca. The area pellucida was cut out from the area opaca in L-15 medium under the dissecting microscope using fine iridectomy scissors.

1. Intact embryos

Two different techniques were utilized to dissociate the intact blastoderms; these are illustrated in a flow chart in Figure 1. Approximately 50 embryos were pooled and transferred to a thick walled test tube (13 mm inner diameter x 100 mm length) containing PCS.

Figure 1. Procedures for the dissociation of intact blastoderms.



Approximately 50 intact blastoderms

Wash with PCS

Wash with 4°C CMF PCS

-or-

Add 2 ml 1 mM EDTA in CMF PCS + 100 units DNase

Incubate 15 min on ice

Resuspend in 2 ml fresh EDTA in CMF PCS + 100 units DNase

Incubate 30 min on ice

Wash and resuspend in 3 ml 4°C PCS + 150 units DNase

Pipette to dissociate

Divide into 2 test tubes

Centrifuge 8 min at 19 x g

Resuspend pellet in 1 ml 4°C PCS + 50 units DNase

Centrifuge 8 min at 19 x g

Resuspend pellet in 1 ml 4°C PCS + 50 units DNase

Centrifuge 7 min at 19 x g

Resuspend pellet in 4°C PCS to 3×10^6 cells/ml

Add 2 ml trypsin (300 units/embryo) in CMF PCS + 100 units DNase

Incubate 15 min on ice

Resuspend in 2 ml fresh trypsin in CMF PCS + 100 units DNase

Incubate 30 min at 37°C

Wash and resuspend in 3 ml 4°C PCS + 150 units DNase

Pipette to dissociate

Divide into 2 test tubes

Centrifuge 8 min at 19 x g

Resuspend pellet in 1 ml 4°C PCS + 50 units DNase

Centrifuge 8 min at 19 x g

Resuspend pellet in 1 ml 4°C PCS + 50 units DNase

Centrifuge 7 min at 19 x g

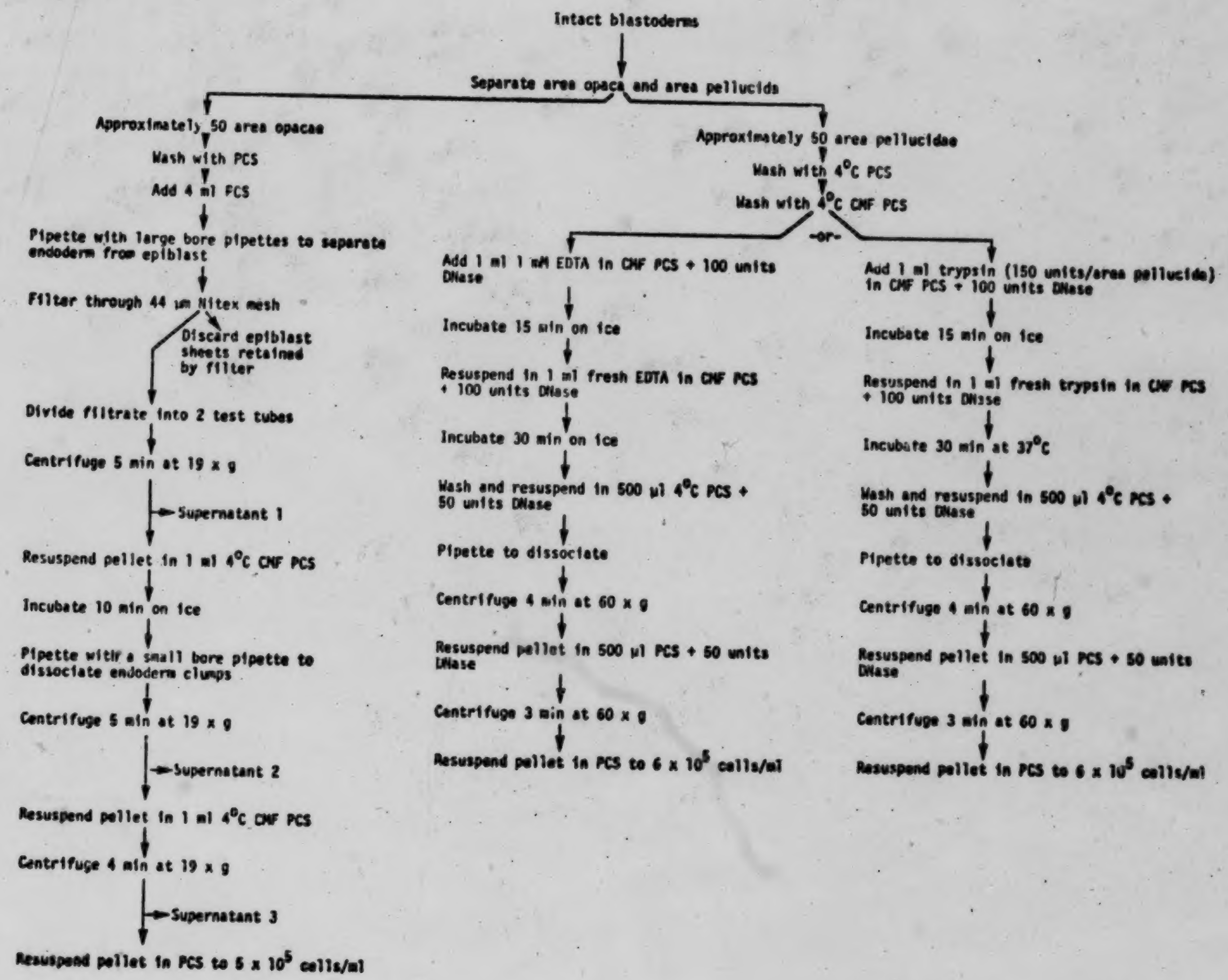
Resuspend pellet in 4°C PCS to 3×10^6 cells/ml

They were rinsed three times with CMF PCS at the temperature of melting ice (4°C). The CMF PCS was removed and the embryos were resuspended in 2 ml of either CMF PCS with 1 mM EDTA or CMF PCS containing 300 units trypsin/embryo. In both cases, DNase was added at a concentration of 100 units/ml and the tubes were incubated on ice for 15 minutes. Following the incubation period, the suspending solution was removed and replaced with 2 ml of the same solution containing 100 units DNase. If the dissociation medium was EDTA the embryos were incubated for 30 minutes on ice; if trypsin was being used the embryos were incubated 30 minutes in a 37°C water bath. The test tubes were shaken gently periodically during the incubation. At the end of the incubation the embryos were washed once with cold PCS and resuspended in 3 ml cold PCS containing 50 units/ml DNase. Dissociation was effected by pipetting the tissue with pipettes of decreasing bore sizes (approximately 4, 2 and 1 mm) a total of about 25 times. The resultant suspensions were divided in half and centrifuged at 19 x g (setting #1 in a clinical centrifuge) for 8 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml cold PCS with 50 units DNase by pipetting one or two times with the smallest bore pipette. The suspension was washed with cold PCS and centrifuged two more times. The final cell pellet was resuspended in PCS to a concentration of approximately 3×10^6 cells/ml. This suspension was maintained at the temperature of melting ice until it was used in reaggregation experiments. This was in usually less than 30 minutes.

2. Area opacae

Suspensions of area opaca endoderm cells (AOEn) were prepared as described by Milos et al. (1979a). This technique relies on the differential dissociability of the area opaca to separate the AOEn from the overlying epiblast cells. The dissociation method is illustrated in Figure 2. Approximately 50 area opacae were pooled and washed three times with room temperature PCS in a thick walled test tube and resuspended in 4 ml PCS. In some cases 50 units/ml DNase were included in this and all subsequent steps, however, the inclusion of the enzyme did not appear to have any effect on the agglutinability of the cells in the subsequent agglutination assay. The tissue was dissociated in PCS by pipetting about 25 times with two pipettes of decreasing bore size (approximately 4 mm and 2 mm). The resulting suspension was filtered twice through 44 µm Nitex nylon mesh (B. & S. H. Thompson & Co., Montreal). The relatively large sheets of epiblast which were not dissociated by this technique were retained on top of the mesh while the endoderm cells which were single or in small clumps passed through. The filtrate was divided into two test tubes with approximately 2 ml in each and centrifuged 5 minutes at 19 x g. The cell pellet was resuspended in 1 ml cold (4°C) CMF PCS and incubated for 10 minutes on ice. The dissociation was completed by pipetting the suspension three to five times with a flamed Pasteur pipette (approximate diameter 0.8 mm). The suspension was centrifuged 5 minutes at 19 x g, the pellet was resuspended in 1 ml CMF PCS by gentle shaking

Figure 2. Procedures for the dissociation of area opacae and area pellucidae.


Intact blastoderms
Separate area opaca and area pellucida
Approximately 50 area opacae
Wash with PCS
Add 4 ml FCS
Pipette with large bore pipettes to separate endoderm from epiblast
Filter through 44 µm Nitex mesh
Discard epiblast sheets retained by filter
Divide filtrate into 2 test tubes
Centrifuge 5 min at 19 x g
Supernatant 1
Resuspend pellet in 1 ml 4°C CMF PCS
Incubate 10 min on ice
Pipette with a small bore pipette to dissociate endoderm clumps
Centrifuge 5 min at 19 x g
Supernatant 2
Resuspend pellet in 1 ml 4°C CMF PCS
Centrifuge 4 min at 19 x g
Supernatant 3
Resuspend pellet in PCS to 5 x 10^5 cells/ml
Approximately 50 area pellucidae
Wash with 4°C PCS
Wash with 4°C CMF PCS
-or-
Add 1 ml 1 mM EDTA in CMF PCS + 100 units DNase
Incubate 15 min on ice
Resuspend in 1 ml fresh EDTA in CMF PCS + 100 units DNase
Incubate 30 min on ice
Wash and resuspend in 500 µl 4°C PCS + 50 units DNase
Pipette to dissociate
Centrifuge 4 min at 60 x g
Resuspend pellet in 500 µl PCS + 50 units DNase
Centrifuge 3 min at 60 x g
Resuspend pellet in PCS to 6 x 10^5 cells/ml
Add 1 ml trypsin (150 units/area pellucida) in CMF PCS + 100 units DNase
Incubate 15 min on ice
Resuspend in 1 ml fresh trypsin in CMF PCS + 100 units DNase
Incubate 30 min at 37°C
Wash and resuspend in 500 µl 4°C PCS + 50 units DNase
Pipette to dissociate
Centrifuge 4 min at 60 x g
Resuspend pellet in 500 µl PCS + 50 units DNase
Centrifuge 3 min at 60 x g
Resuspend pellet in PCS to 6 x 10^5 cells/ml

and recentrifuged at 19 x g for 4 minutes. If the suspension was to be examined immediately for lectin-mediated agglutination, the cells were suspended in PCS to a final concentration of approximately 5 x 10^5 cells/ml and kept at the temperature of melting ice. If the cells were to be further treated with enzymes, the nature of the final resuspending medium was as described in section D.

3. Area pellucidae

Area pellucidae (AP) were dissociated by one of two different procedures; one employed EDTA, and the other used trypsin in the dissociation medium (Figure 2). Usually, the pellucidae obtained on any one day were divided in half and both halves were dissociated simultaneously by each dissociation procedure. Approximately 50 pellucidae were transferred to a thin walled test tube (11 x 100 mm) and rinsed two times with cold PCS. This was followed by four washes of cold CMF PCS. The tissue was resuspended in 1 ml of either CMF PCS with 1 mM EDTA or CMF PCS with 150 units trypsin/pellucida. DNase (100 units/ml) was added to both tubes which were incubated on ice for 15 minutes. The solution was removed and replaced with 1 ml of the same solution containing 100 units DNase. The embryos in the EDTA solution were incubated for 30 minutes on ice while those in trypsin were incubated for 30 minutes at 37°C. Following the incubation, both samples were washed once with about 3 ml cold PCS and each resuspended in 500 µl cold PCS with 50 units DNase. The pellucidae were dissociated by pipetting about 25 times with two pipettes of decreasing bore size (approximately 0.8 and 0.2 mm). The suspension was centrifuged 4 minutes at 60 x g (setting #2), resuspended in 500 µl cold PCS with 50 units DNase and recentrifuged for 3 minutes at 60 x g. When the cells were to be examined immediately for lectin-mediated agglutination the pellet was resuspended in PCS to a final concentration of approximately 6 x 10^5 cells/ml and kept on ice. If the cells were to be treated further, the suspending medium is described in the following section.

D. Enzyme Treatments of Dissociated Cells

1. Neuraminidase

Neuraminidase treatment of dissociated cells is required to render them agglutinable with SBA (Zalik and Cook, 1976; Phillips and Zalik, 1978). Instead of resuspending the cells in PCS after the final centrifugation, cell suspensions of either AOEn or AP were resuspended in 1 ml CMF PCS. The suspension was equally divided into test tubes and 500 µl PCS was added to each tube. DNase (50 units) was added to both tubes and 20 µl NaNase (10 units) was added to one tube; the other tube served as a control. Both samples were incubated for 20 minutes at 37°C with occasional shaking. At the end of the incubation period both tubes were centrifuged (5 minutes at 19 x g for AOEn or 4 minutes at 60 x g for AP) and resuspended in PCS. The final resuspension volumes were adjusted such that there were approximately 5 x 10^5 AOEn cells/ml or 6 x 10^5 AP cells/ml. These suspensions were maintained on ice and assayed for SBA-mediated agglutination.

2. Trypsin

Area pellucida cells were dissociated with either EDTA or trypsin; cells obtained from both procedures were assayed for lectin-mediated agglutinability. Area opaca endoderm cells, however, were always dissociated in CMF PCS. In order to examine the effect of trypsinization on agglutination of AOEn cells, freshly dissociated cells were incubated with trypsin before agglutination was assayed. After the final centrifugation in the dissociation procedure, AOEn cells were resuspended in 4 ml CMF PCS and divided equally into 4 tubes. DNase was added to each of the tubes at a concentration of 100 units/ml. Two of the tubes served as controls; one control was incubated on ice and the other was incubated at 37°C. Trypsin was added to the third tube in a 20 µl aliquot of CMF PCS. The final concentration of trypsin was 50 units/area opaca, i.e. if 100 area opacae were dissociated, it was assumed that there were cells from 25 area opacae in each tube after dissociation, thus in this case 1,250 units of trypsin were added to the tube. In some cases, SBTI was added to the trypsin sample at a 1:1 weight relation to trypsin. SBTI alone was added to the fourth tube at the same concentration it was added to the trypsin sample. The four tubes were incubated for 20 minutes, the 4°C control on ice and the other three at 37°C. Following the incubation, all four tubes were centrifuged at 19 x g for 5 minutes and the cell pellet was resuspended in PCS. The final cell concentration was adjusted to approximately 5 x 10^5 cells/ml in each tube. The cells were kept on ice and assayed for lectin-mediated agglutinability.

E. Agglutination Assay

The agglutination response was quantified by monitoring the disappearance of single cells from a cell suspension with a Coulter Counter model TA II particle counter (Coulter Electronics). This particular model of counter can count and size particles as they pass through an aperture of a known diameter. A current also passes through the aperture between electrodes placed on either side of the aperture. Particles are suspended in a conductive medium, in this case PCS, and drawn through the aperture by means of a vacuum pump connected to the system. As a particle passes through the aperture it alters the resistance, causing a current flow which is proportional to the volume of the particle. These pulses are scaled and recorded in one of 16 channels depending on the size of the pulse (Coulter Counter model TA II Reference Manual). When the machine is calibrated with particles of a known diameter it is possible to count a population of particles and determine the number and size distribution of the particles. Although the current pulse is proportional to the particle volume, it is more convenient to express particle size as diameter. In order to do this it is necessary to assume that the particles are spherical. While this is not always the case for a population of cells, the Coulter Reference Manual states that there is an averaging effect due to the fact that in a large population the particles will be passing through the aperture in all possible orientations, thus making the assumption valid.

In this investigation the Coulter Counter was fitted with a 280 μm aperture tube and calibrated with 20.18 μm polystyrene microspheres (Coulter Electronics). The diameter range of particles which were counted in each of the channels were as follows:

Channel	Particle diameter (μm)
1	4.0- 5.0
2	5.0- 6.4
3	6.4- 8.0
4	8.0- 10.1
5	10.1- 12.7
6	12.7- 16.0
7	16.0- 20.2
8	20.2- 25.4
9	25.4- 32.0
10	32.0- 40.3
11	40.3- 50.8
12	50.8- 64.0
13	64.0- 80.6
14	80.6-101.6
15	101.6-128.0
16	>128.0

Each subsequent channel represents a doubling of the particle volume. Channel 16 is an open ended channel, that is, all particles greater than 128 µm are counted in channel 16. Counts in channels 1 and 2 were not routinely recorded; counts in channel 1 may be affected by electrical noise and both channels accumulated counts due to small particles of cellular debris.

In practice, aliquots of the cell suspension were diluted with Millipore filtered PCS such that the total number of counts accumulated in channels 3 to 16 in a 2 ml sample was less than 3,630. This keeps error due to coincidence (the passage of more than one particle at a time through the aperture) to less than 5% (Coulter Counter model TA II Reference Manual). Samples were diluted with 25 ml PCS and 10 ml of the sample were counted with the counter. The time required to count this volume with the 280 µm aperture tube was 32.5 seconds.

The counter was equipped with a Population Accesory which calculated and provided a printout of the number of particles accumulated in each channel. The channels which contained counts due to single cells were determined by measuring the diameters of single cells under the microscope.

Agglutination assays were performed within the well created by glueing, with silicone sealant, a 10 mm inner diameter x 2 mm high plastic ring to the bottom of a 25 ml plastic vial (Coulter Electronics) (Figure 3). This assembly was reusable and could withstand repeated washing. PCS, with or without lectin, and where applicable, hapten, was added to the well in a total volume of 130 µl. A 20 µl aliquot of the cell suspension was added to the

Figure 3. Vial assembly used in the agglutination assay. Actual size.

fluid in the well and the vial was capped and rapidly swirled a few times to disperse the cells. The vial was then incubated on a gyratory shaker (New Brunswick Scientific) at 135 rpm. After a 20 minute incubation period the vial was removed and the extent of agglutination was assessed visually under a dissecting microscope. Agglutination was roughly scored on a scale of 0, +, ++, +++ and ++++ where 0 indicates that no agglutination occurred, + indicates that 0 to 30% of the cells agglutinated, ++ indicates 30 to 60% agglutination, +++ indicates 60 to 90% agglutination, and ++++ indicates that over 90% of the cells agglutinated (Phillips and Zalik, 1978). After the visual assessment the cell suspension was diluted with 25 ml cold (4^{o}C) PCS added from an Oxford pipettor. The diluted cell suspension was stirred gently one or two times with a metal spatula and counted with the Coulter Counter.

A major problem inherent in an assay for lectin-mediated agglutination of cells dissociated from early chick embryos is that these cells undergo rapid reaggregation. In this report the term aggregation is used to define the spontaneous clumping of cells which occurs in the absence of any exogenous ligand or lectin. The clumping of cells which is induced by the addition of an exogenous lectin is termed agglutination. Embryonic chick cells have been shown to reaggregate rapidly even at room temperature in PCS (Milos et al., 1979a; Cook et al., 1979). With this in mind, it was necessary to design an assay system which could discriminate between the two processes, or barring that, a system which could reduce reaggregation to a minimum while not affecting agglutination. It was posible to capitalize on the fact that aggregates formed in PCS at room temperature are quite loosely adherent and sensitive to high shear forces. Incubating the vials at 135 rpm produces enough shear forces to minimize cell aggregation in the absence of lectin but does not appear to inhibit agglutination when lectin is present. A rotation speed of 100 rpm is more conducive to aggregation under these experimental conditions. By comparing visual assessment of agglutination with values obtained from the Coulter Counter it was apparent that aggregates were more susceptible to dissociation by the dilution with cold saline than were cell clumps which had been formed in the presence of lectin. The dilution technique, therefore, provided an effective method of selectively reducing aggregation. As an additional means to distinguish aggregation from agglutination the vials were inoculated and counted as follows. Twenty vials were prepared by dispensing PCS and lectin into the wells to a total volume of 130 μl. In most cases, there was one control vial without lectin prepared for every four vials with lectin. A 20 μl aliquot of the cell suspension being tested was diluted in 25 ml cold PCS and counted with the Coulter Counter to determine the number of single cells initially present. The suspension had been adjusted previously so that the 20 μl aliquot contained approximately 1×10^4 AOEn or 1.2×10^4 AP cells. At time zero (T_0) the first vial was inoculated with cells and placed on the rotary shaker. The rest of the vials were inoculated at 60 second intervals (T_1, T_2, . . . T_{19}). At T_{20} the first vial was removed, agglutination was checked visually and the sample was diluted and counted. The whole operation; visual check, dilution, and counting could be performed in just under 60 seconds, thus each vial could be counted after exactly 20 minutes of incubation. An estimate of the extent of aggregation could be made by comparing the number of single cells present at T_{20} in the sample which did not contain lectin with the number of single cells present in the initial sample counted at T_0. The per cent aggregation was calculated by the following formula:

$$\%\ \text{Aggregation} = \frac{\left[\begin{array}{l}\text{Number of single}\\ \text{cells at } T_0\end{array}\right] - \left[\begin{array}{l}\text{Number of single}\\ \text{cells at } T_{20} \text{ in}\\ \text{absence of lectin}\end{array}\right]}{\text{Number of single cells at } T_0} \times 100$$

Under the experimental conditions the aggregation was usually less than 20%. In order to determine the per cent agglutination it was assumed that this base level amount of aggregation was present in all samples in the presence or absence of lectin. If fewer single cells were present in the T_{20} lectin sample than the lectin free control at T_{20}, these cells were assumed to have been agglutinated. The per cent agglutination, therefore, could be calculated separately from aggregation by comparing the number of single cells present at T_{20} in the lectin free control with the lectin treated sample.

$$\% \text{ Agglutination} = \frac{\left[\begin{array}{l}\text{Number of single}\\ \text{cells at } T_{20} \text{ in}\\ \text{lectin free sample}\end{array}\right] - \left[\begin{array}{l}\text{Number of single}\\ \text{cells at } T_{20} \text{ in}\\ \text{lectin sample}\end{array}\right]}{\begin{array}{l}\text{Number of single cells at } T_{20} \text{ in lectin}\\ \text{free sample}\end{array}} \times 100$$

This calculation provides an estimate of the lectin-mediated agglutination over the spontaneous aggregation.

F. Protease Assay

Intact area opacae, dissociated AOEn cells, and cell free supernatants were assayed for presumed protease activity by two methods. Azocoll (Calbiochem) is a general protease substrate consisting of an insoluble powdered cowhide attached to a red dye. Cleavage of peptide linkages by a protease results in the release of the dye into the suspending medium. The release can be quantified by measuring the absorbance at 520 nm with a spectrophotometer and comparing the values to protease standards.

Approximately 5 x 10^5 freshly dissociated AOEn cells were incubated with 25 mg Azocoll in a total volume of 5 ml PCS for 15 minutes at 37°C. Pooled supernatants 1 and 2 (see Figure 2) from AOEn cell dissociations were centrifuged at 16,000 x g for 20 minutes in a Sorvall RC-2 centrifuge to pellet any cells and cell fragments. The supernatants were assayed with Azocoll similarily to the AOEn cell suspensions. Usually 1 or 2 ml supernatant were added to PCS to a total of 5 ml. The incubation with Azocoll was terminated by filtering through Whatman #1 filter paper. The absorbance of the filtrate was measured at 520 nm. Blanks were prepared by incubating Azocoll and PCS similarily to the test samples. A standard protease activity curve was calculated by incubating weighed samples of trypsin with Azocoll. The effectiveness of SBTI as a trypsin inhibitor was also determined with this assay.

The casein digestion method described by Kunitz (1947) was also used to test for protease activity. A stock solution of casein was prepared by dissolving 1 g casein (Hammersten), (Schwartz/Mann) in 100 ml PCS by heating in a boiling water bath for 15 minutes. This was cooled and stored at 5°C for no more than one week. Approximately 1 x 10^6 AOEn cells, 10 intact area opacae or cell free supernatant were placed in a conical centrifuge tube in a total volume of 1 ml PCS. The incubation was started by adding 1 ml of the casein solution which had been warmed to 37°C and placing the tubes in a 37°C water bath. Cells and supernatants were incubated for 20 minutes and intact area opacae were incubated for 60 minutes. The incubation was terminated by adding 3 ml 5% (w/v) trichloroacetic acid (TCA) and vortexing the tubes. The precipitates were allowed to settle for 30 minutes and the tubes were centrifuged for 30 minutes at 16,000 x g. Blanks were prepared by mixing 1 ml casein with 3 ml TCA and then adding the sample in 1 ml PCS. The absorbance of the supernatants was read at 280 nm with the spectrophotometer and corrected for the blanks. Trypsin standards were also assayed with this method.

G. Aggregation of Blastoderm Cells

The effect of low concentrations of lectin on the ability of dissociated cells to reaggregate and undergo subsequent morphogenesis was examined. Whole blastoderms were dissociated under sterile conditions using either EDTA or trypsin. The cells were resuspended in PCS at a final concentration of 3 x 10^6 cells/ml. L-15 medium supplemented with 0.1% (w/v) bovine serum albumin (BSA), (Sigma) and 20 µg/ml gentamycin sulfate were added to 10 ml Erlenmeyer flasks which had been siliconized and autoclaved. Lectin and/or hapten at the appropriate concentrations were also added. The total volume of medium in the flasks was 2.5 ml. If RCA was the lectin being tested the medium used was the special formula L-15 which contained glucose instead of galactose as the latter sugar may act as a hapten for RCA. The cell suspension was added to the flask in a 100 µl aliquot (final concentration approximately 1.2 x 10^5 cells/ml) and the flasks were incubated in a gyratory incubator at 37°C and 70 rpm. After 1 hour the rotation speed was increased to 100 rpm. After 20 to 24 hours the medium in the flasks was changed. Approximately 2 ml medium were removed and replaced with 2.5 ml fresh L-15 medium supplemented with 15% (v/v) fetal calf serum (FCS), (Gibco) and 20 µg/ml gentamycin sulfate. Alternatively, the aggregates were removed from the flask in as small a volume as possible and transferred to a new flask containing 3 ml L-15 medium with FCS and gentamycin. The aggregates were cultured for up to 3 additional days. The medium was usually changed once more during this period. At the end of the experiment the aggregates were harvested and fixed in Bouin's fixative for at least 24 hours. The aggregates were dehydrated in a series of alcohols and embedded in paraffin. Sections were cut at 5 to 7 μm and the deparaffinized sections were stained with Haematoxylin and Eosin.

RESULTS

A. Cell Suspensions

All of the dissociation techniques utilized in this investigation provided cell suspensions with more than 80% single cells. The remainder of the cells was present as pairs and small clumps generally containing less than ten cells. The viability of the cells was high; more than 95% of the cells remained unstained in 0.1% trypan blue, and when cultured at 37°C the cells reaggregated and underwent differentiation.

The greatest variability between different cell suspensions was in the cleanliness of the final suspension. A certain amount of cell lysis is unavoidable in any dissociation technique which requires mechanical disruption of a tissue. Cells from primitive streak stage blastoderms are quite large and may contain large quantities of intracellular yolk platelets, particularly the extraembryonic endoderm cells (Bellairs, 1963). These yolk platelets are a major portion of the contaminating debris. Nuclei, cytoplasmic organelles, and membrane vesicles also contribute to the debris. The dissociation techniques used in this study were designed with an emphasis on obtaining relatively clean cell preparations with a minimal number of experimental manipulations. Washing the suspensions with clean saline and low speed centrifugation helped to remove a large portion of the subcellular debris, however, excessive centrifugation tended to clump the cells together, thus necessitating additional pipetting which caused more cell lysis. In the dissociation techniques, therefore, a point was reached where it was determined that further washing of the cells did not improve the quality of the cell suspensions.

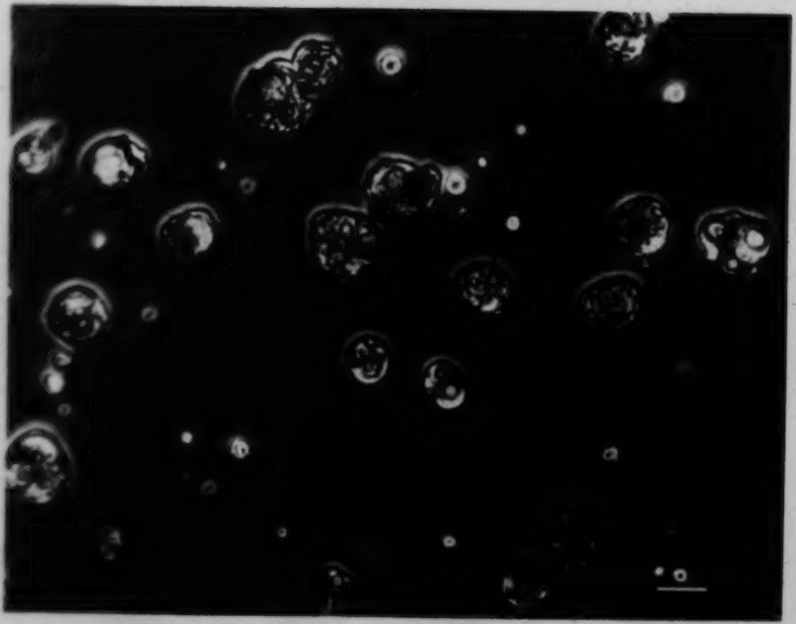
Generally, in regard to cleanliness, the best cell preparations were those obtained after trypsin dissociation of area pellucida, followed by those obtained by EDTA dissocation of the same embryonic area. Cell suspensions of endoderm from the area opaca and suspensions from whole blastoderms usually contained more debris. This is not surprising as AOEn cells are larger and contain more intracellular yolk (Bellairs, 1963). Bellairs also has reported that large yolk spheres are intercalated between endoderm cells of the area opaca. Microscopic examination of sectioned blastoderms has confirmed these observations. Blastoderms were washed extensively in PCS to remove most of the adhering yolk before dissociation but it would have been impossible to remove all of the yolk without a concomitant loss of a considerable number of cells.

Suspensions of AOEn cells consisted primarily of large cells which contained numerous yolk platelets (Figure 4a). Occasionally, small clumps of contaminating epiblast cells were observed; these cells were characterized by being smaller, containing less yolk, and often exhibiting extensive protrusions of hyaline blebs. Milos et al. (1979a) have reported that AOEn cells dissociated by the same technique employed in this study had an average

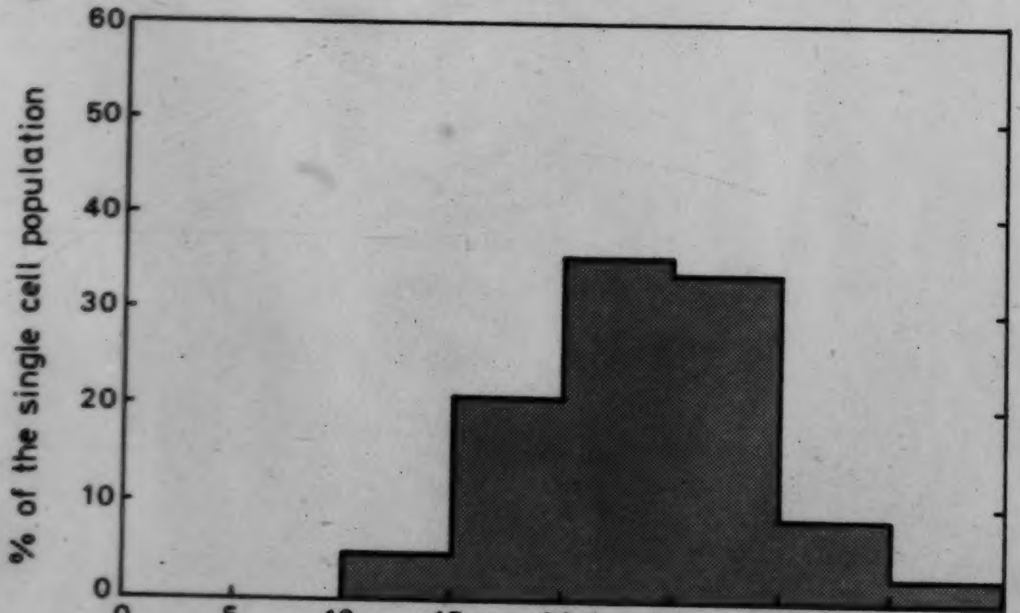
Figure 4a. Dissociated area opaca endoderm cells suspended in PCS. Note the large numbers of yolk platelets within the cells and the presence of lobopodia on some of the cells (arrows). Single yolk platelets are also present in the medium (*). The bar is 25 µm.

Figure 4b. Size range of dissociated area opaca endoderm cells suspended in PCS. The diameters of 138 cells were measured under the microscope. The average cell diameter was 24 µm.
(From Milos et al., 1979a).

A



B



% of the single cell population
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40
Cell Diameter (μm)

diameter of 24 μm (Figure 4b). When the Coulter Counter is fitted with a 280 μm aperture tube approximately 90% of the single cell population are counted in channels 7, 8 and 9. This corresponds to a size range of 16 to 32 μm.

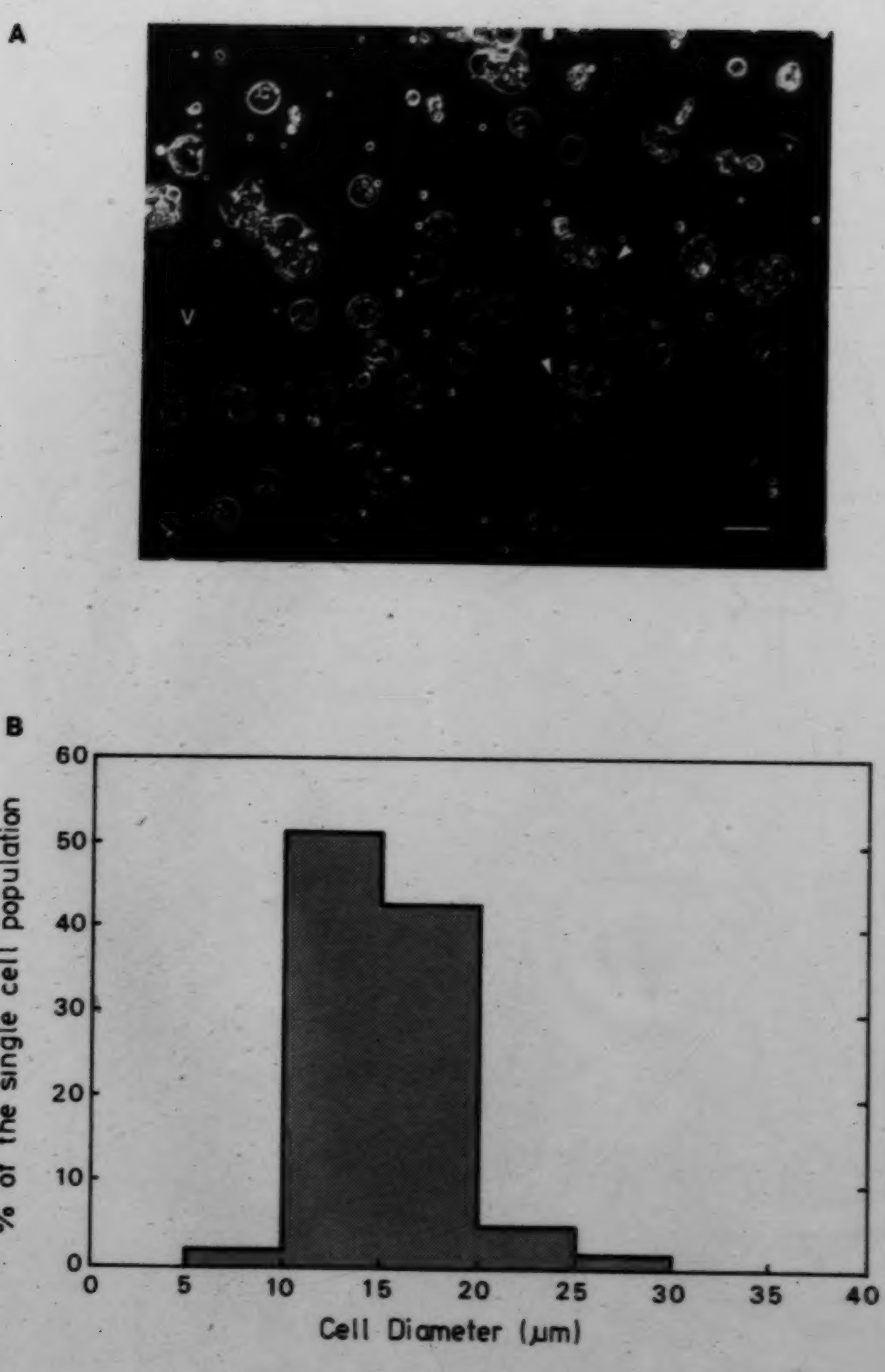
Cell suspensions obtained from the area pellucida contained cells derived from all three germ layers. The cells were generally smaller than AOEn cells and exhibited different morphologies. Some cells contained numerous yolk platelets similarily to the AOEn cells but the majority of them contained very little yolk (Figure 5a). Large numbers of the cells exhibited the extensive blebbing that was observed in the contaminating epiblast cells in AOEn preparations. The average diameter of AP cells was 15 μm and the range of cell sizes was from 9 to 29 μm (Figure 5b). Approximately 85% of the single cells were in the size range of 12.7 to 20.2 μm which corresponds to channels 6 and 7 of the Coulter Counter. Both the cell morphologies and size range of AP cells were similar to EDTA and trypsin dissociated preparations.

The counts accumulated in channels 6 to 9 were totalled to determine the number of single cells present in a dissociation of whole blastoderms.

Unfortunately, some of the contaminating yolk platelets were in the same size range as the single cells. The numbers obtained in channels 6 to 9, therefore, were a slight overestimate of the actual number of single cells.

Figure 5a. Trypsin dissociated area pellucida cells suspended in PCS. Note the smaller cell size compared to Figure 4a and the presence of numerous hyaline lobopodia (arrows). Generally, the cells contain smaller numbers of intracellular yolk platelets than area opaca endoderm cells. There are numerous highly refractile yolk platelets present within the saline (*). A membrane vesicle is also present (v). The bar is 25 μm.

Figure 5b. Size range of dissociated area pellucida cells. The diameters of 213 cells dissociated with EDTA and 213 cells dissociated with trypsin were measured under the microscope. The pooled data was plotted as there was no significant difference in the size range of cells dissociated by either method. The average cell diameter was 15 μm.


A
V
B
% of the single cell population
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40
Cell Diameter (µm)

B. Agglutination Response

The values for agglutination which are reported here represent the per cent disappearance of single cells from a sample which could be attributed solely to the presence of the lectin. The disappearance of single cells which was due to spontaneous cell reaggregation could be effectively removed from the calculation as described in the Materials and Methods.

Area opaca endoderm and area pellucida cells exhibited a similar agglutinability with Con A, WGA, RCA, and SBA as it has been reported for cells dissociated from whole blastoderms (Zalik and Cook, 1976; Phillips and Zalik, 1978). Quantification of the agglutination response, however, has revealed differences which were undetectable with the visual assessment of agglutination used previously.

Agglutination of endodermal cells from the area opaca, as monitored by the Coulter Counter, is portrayed graphically in Figures 6 to 9. The ordinate represents the size range or channel number and the abscissa represents the number of particles counted. Figure 6 shows the agglutination mediated by 10 μg/ml WGA. In the control (no lectin present) there is a peak particle count in channels 7, 8, and 9; this corresponds to the single cell population. Counts in lower channels represent cellular debris and small yolk particles; counts in the higher channels, 10, 11, and 12, are due to large cells and yolk spheres, and small clumps of cells. When

Figure 6. Particle counts of area opaca endoderm cells incubated in the absence (-O-) or presence of 10 μg/ml WGA (--●--). Cells were incubated for 20 minutes at room temperature, diluted with cold PCS and counted with the Coulter Counter. The counts in channels 7, 8, and 9 correspond to the single cell population. Note the decrease in the number of particles counted in these channels in the presence of WGA. In the particular experiment illustrated here there is a 56% decrease in the number of particles in channels 7 to 9 in the presence of WGA.

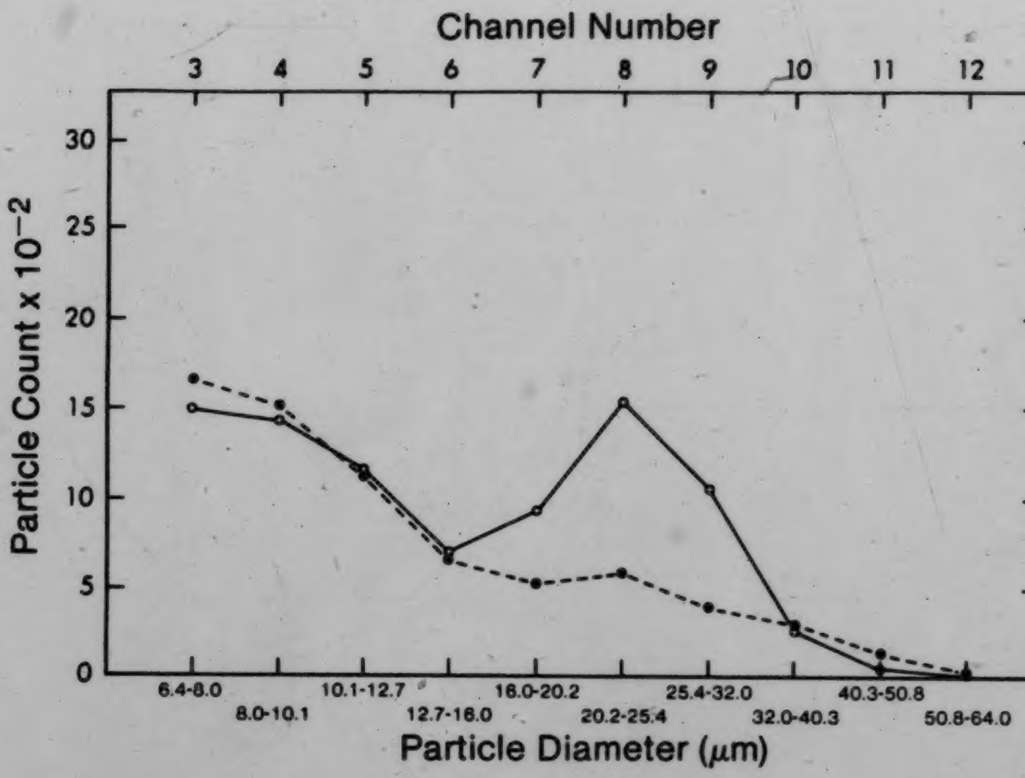

Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^{-2}
30
25
20
15
10
5
0
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)

Figure 7a. Particle counts of area opaca endoderm cells in the absence (-0-) or presence of 100 μg/ml SBA (--●--). Cells were incubated for 20 minutes, diluted with cold PCS, and counted with the Coulter Counter. There is essentially no difference in the particle counts of the two samples indicating that SBA does not cause agglutination of these cells.

Figure 7b. Particle counts of neuraminidase treated area opaca endoderm cells in the absence (-0-) or presence of 100 μg/ml SBA (--●--). Cells were pretreated with 10 units/ml neuraminidase for 20 minutes at 37°C, washed, and incubated for 20 minutes at room temperature in the absence or presence of SBA. The samples were diluted with cold PCS and counted with the Coulter Counter. There is an SBA-mediated decrease in the number of particles counted in channels 7, 8, and 9 which accumulate counts due to single cells. In the experiment illustrated here, this is a 60% decrease.

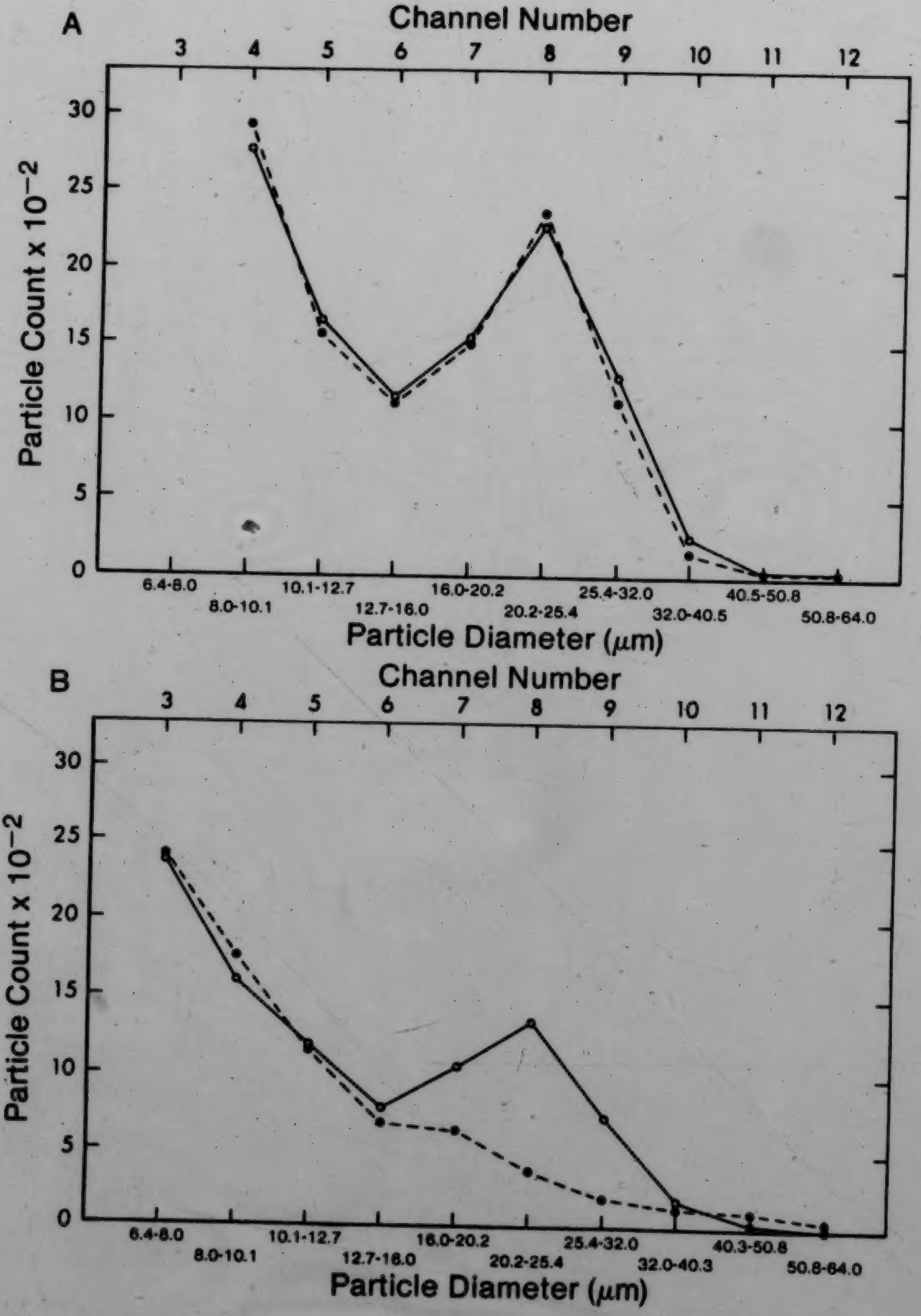

A
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^{-2}
30
25
20
15
10
5
0
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.5
40.5-50.8
50.8-64.0
Particle Diameter (μm)
B
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^{-2}
30
25
20
15
10
5
0
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)

Figure 8a. Particle counts of area opaca endoderm cells incubated in the absence (-0-) or presence of 10 μg/ml RCA (--●--). Cells were incubated for 20 minutes at room temperature, diluted with cold PCS, and counted with the Coulter Counter. In the experiment illustrated here, there is a 70% decrease in the number of particles counted in channels 7, 8, and 9 in the RCA treated sample. RCA also caused a 33% decrease in the number of particles counted in channels 3 to 6.

Figure 9b. Particle counts of supernatants from area opaca endoderm cell suspensions incubated in the absence (-0-) or presence of 10 μg/ml RCA (--●--). The cell suspension was centrifuged to pellet most of the cells and the supernatant was incubated for 20 minutes at room temperature, diluted with cold PCS and counted with the Coulter Counter. Note that the number of counts in channels 7 to 9 is greatly reduced from Figure 8a. In the experiment illustrated here there is a 13% decrease in particles in channels 3 to 6 and a 38% decrease in channels 7 to 9 when RCA is present.

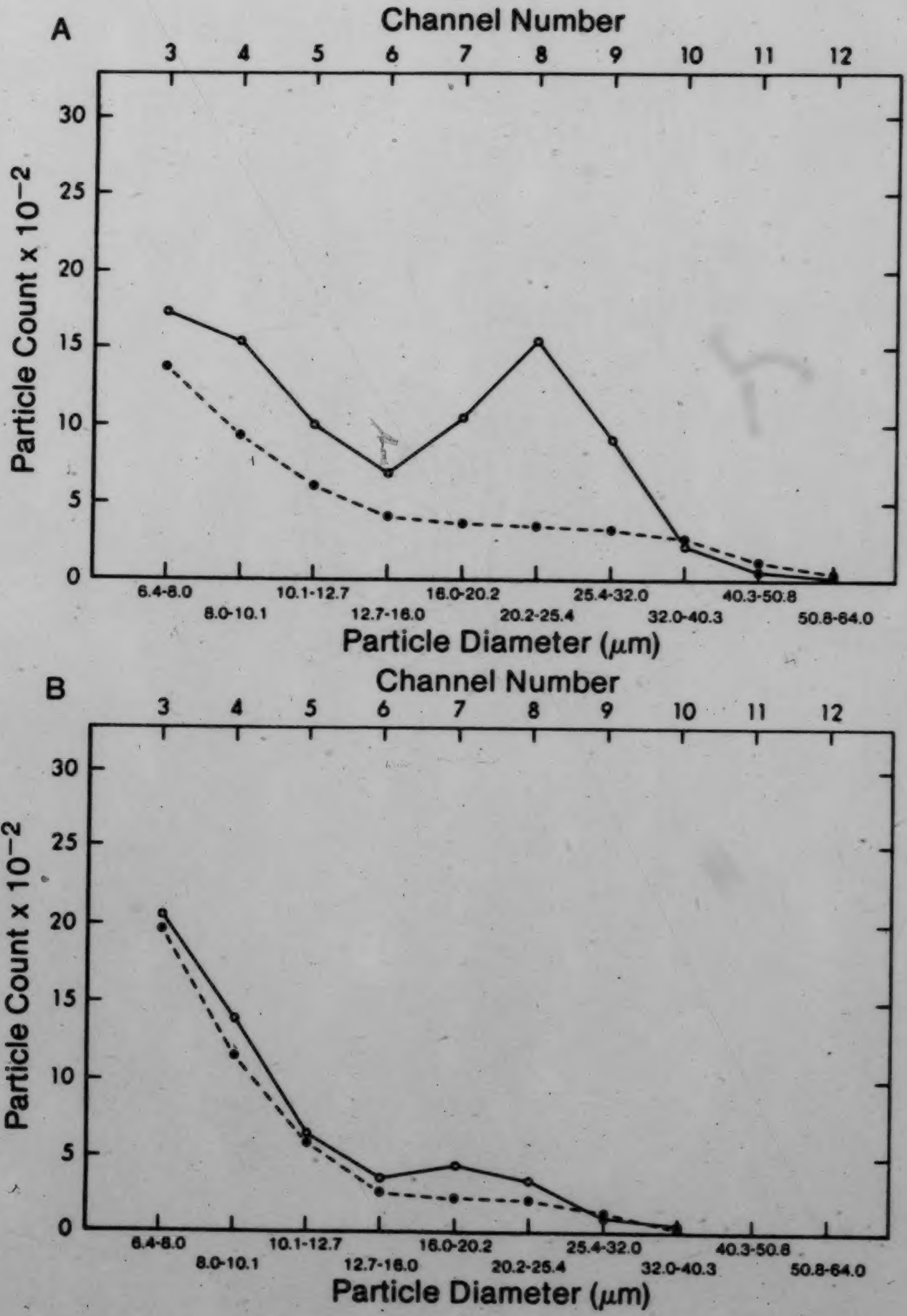
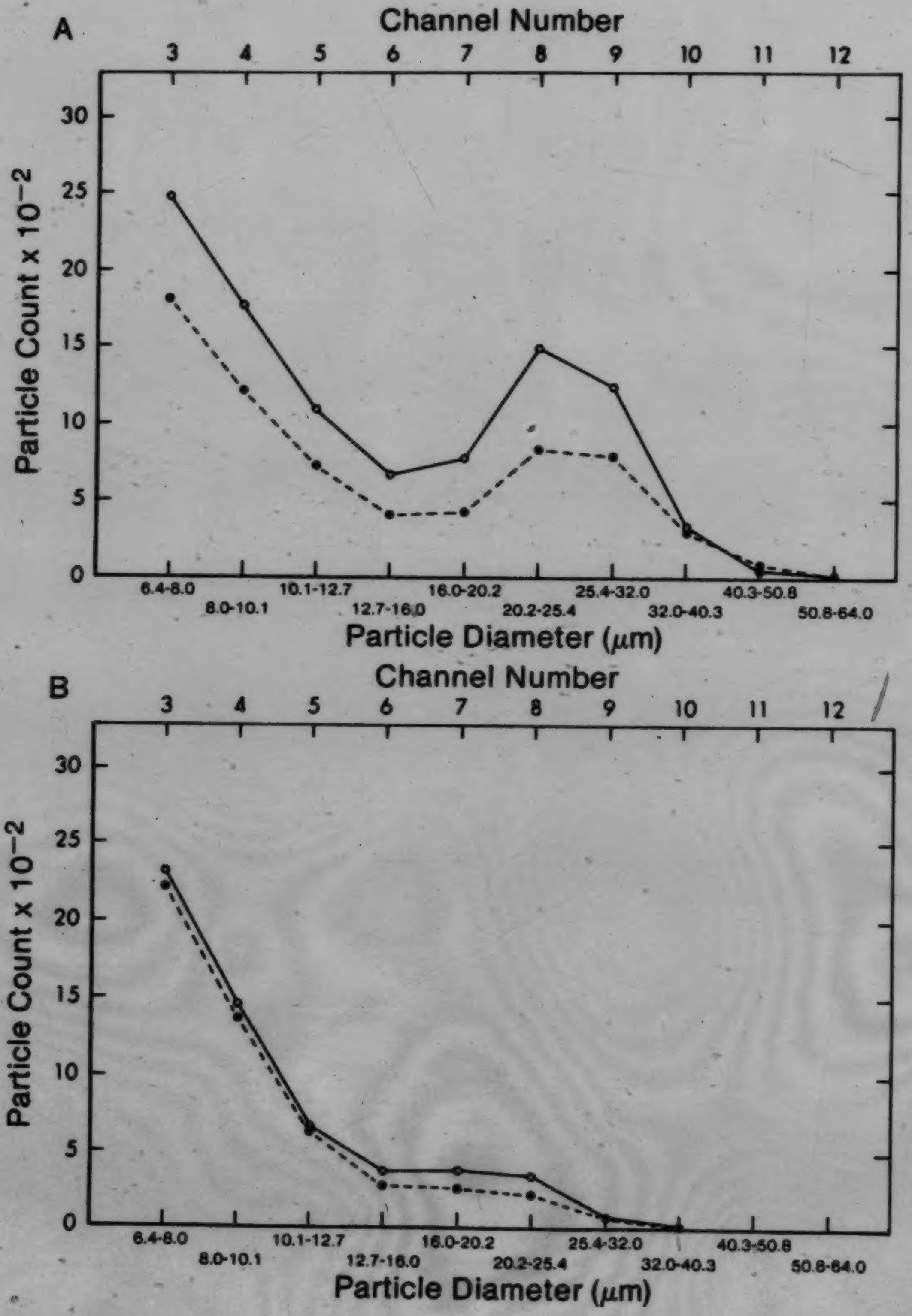

A
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10⁻²
0
5
10
15
20
25
30
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)
B
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10⁻²
0
5
10
15
20
25
30
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)

Figure 9a. Particle counts of area opaca endoderm cells incubated in the absence (-0-) or presence of 12.5 μg/ml Con A (--●--). Cells were incubated for 20 minutes, diluted with cold PCS, and counted with the Coulter Counter. In the experiment illustrated here, Con A mediated a 37% decrease in the number of particles counted in channels 7 to 9, as well as a 31% decrease in the number of particles counted in channels 3 to 6.

Figure 9b. Particle counts of supernatants of area opaca endoderm cell suspensions incubated in the absence (-0-) or presence of 12.5 μg/ml Con A (--●--). The cell suspension was centrifuged at low speed to pellet most of the cells and the supernatant was incubated for 20 minutes at room temperature, diluted with cold PCS and counted with the Coulter Counter. Note the reduction of particles counted in channels 7, 8, and 9 from Figure 9a. In the experiment illustrated here, Con A mediated a 6% decrease in the number of particles counted in channels 3 to 6 and a 30% decrease in the number of particles in channels 7 to 9.


A
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^{-2}
0
5
10
15
20
25
30
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)
B
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^{-2}
0
5
10
15
20
25
30
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)

10 μg/ml WGA is present there is a marked reduction in the number of particles accumulated in channels 7, 8, and 9. The per cent decrease of particles in these channels, or as it is expressed in this report, the per cent agglutination, is 56% in the case shown in Figure 6. There is a small increase in the number of particles accumulated in the larger size channels 11 and 12 which is due to the agglutinating action of the lectin, however, as only a few clumps are formed from a large number of cells the statistical probability of counting a clump is low. Also, the large clumps tend to settle to the bottom of the counting vial, thus they do not pass through the aperture.

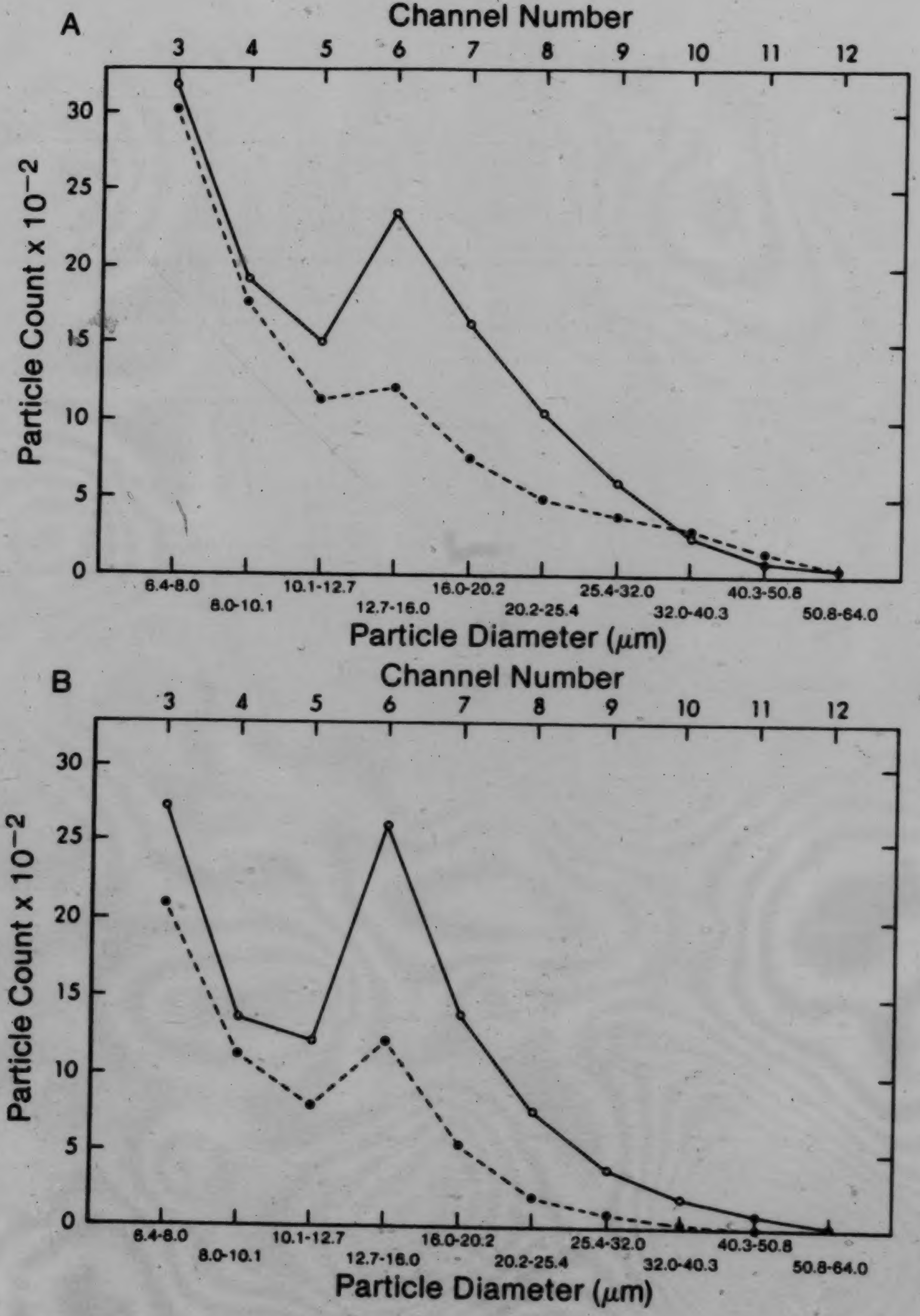
Untreated AOEn cells were not agglutinated by SBA. The response of AOEn cells to 100 μg/ml SBA is shown in Figure 7a. There is essentially no difference between the particle counts in the lectin free control and the sample containing SBA. If dissociated AOEn cells were pretreated with 10 units/ ml NaNase at 37°C for 20 minutes they were rendered agglutinable with SBA (Figure 7b). Agglutination of AOEn cells with RCA and Con A is shown in Figures 8a and 9a respectively. Both of these lectins agglutinated the cells as evidenced by the decreased particle counts in channels 7 to 9 but there was also a significant decrease in the particle counts in channels 3 to 6. This suggests that the subcellular particles are also being agglutinated by these lectins. This response was consistently observed with these two lectins. Microscopic observation of the agglutinated cell clumps formed in the presence of Con A or RCA revealed that small particles were incorporated in between the cells in the clumps. Single cells also often had numerous small particles adhering to their surfaces. The lectin-mediated disappearance of these small particles appeared to require the presence of cells in the suspension. If the majority of the cells was removed from the suspension by low speed centrifugation and the resulting supernatant, which contained the small particles, was examined for its agglutinability with RCA and Con A the disappearance of the small particles was greatly reduced (Figures 8b and 9b). If cells were added back to the supernatant, however, the disappearance of small particles was increased.

Agglutination of AP cells was generally similar to AOEn cells. For purposes of comparison, Figures 10a and 10b show agglutination profiles of trypsin dissociated AP with WGA and Con A. In the case of AP the peak corresponding to the single cells is observed in channels 6 and 7. Con A and RCA also caused a disappearance of subcellular particles from AP suspensions but this was not as obvious as it was with AOEn cells. One obscuring factor was that AP suspensions contained more cells that were counted in channel 5.

Visual assessment of Con A and RCA-mediated agglutination of both AOEn and AP cells revealed an additional difference which set these two lectins apart from WGA and SBA. Both Con A and RCA caused a large proportion of the cells to adhere to the plastic bottom of the vial. Dilution of the sample with PCS at the end of

Figure 10a. Particle counts of trypsin dissociated area pellucida cells incubated in the absence (-O-) or presence of 12.5 μg/ml WGA (--●--). Cells were incubated for 20 minutes at room temperature, diluted with cold PCS, and counted with the Coulter Counter. The majority of single cells is counted in channels 6 and 7. In the experiment illustrated here WGA caused a 48% decrease in the number of particles counted in these two channels. Some cells are also counted in channels 5, 8, and 9, hence, there is also a WGA-mediated decrease in the number of particles accumulated in these channels.

Figure 10b. Particle counts of trypsin dissociated area pellucida cells in the absence (-O-) or presence of 12.5 μg/ml Con A (--●--). Cells were incubated for 20 minutes at room temperature, diluted with cold PCS, and counted with the Coulter Counter. Con A causes a decrease in the number of particles counted in the single cell channels 6 and 7 (55% in this case) as well as a decrease in the number of particles in channels 3 and 4 (20%).


A
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^-2
30
25
20
15
10
5
0
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)
B
Channel Number
3
4
5
6
7
8
9
10
11
12
Particle Count x 10^-2
30
25
20
15
10
5
0
6.4-8.0
8.0-10.1
10.1-12.7
12.7-16.0
16.0-20.2
20.2-25.4
25.4-32.0
32.0-40.3
40.3-50.8
50.8-64.0
Particle Diameter (μm)

the incubation period did not appear to remove these attached cells, thus, they were effectively removed from the population and were not counted with the Coulter Counter. This response was considered as specific lectin-mediated agglutination as it was abolished by the addition of the specific hapten to the incubating medium. Various treatments known to reduce nonspecific adhesion of proteins to plastic were, at best, only partially effective at preventing the adhesion of cells to the substrate. Siliconization of the vials did not affect this lectin-mediated cell attachment. Rinsing the wells with a 2% (w/v) solution of BSA just prior to the agglutination assay or the inclusion of 0.5% (w/v) BSA during the incubation reduced the sticking at low lectin concentrations (less than 2 μg/ml) but was ineffective at higher lectin concentrations. In cases where the BSA reduced sticking, the per cent agglutination was similar to the value for samples in which BSA was not present. This finding also suggests that lectin-mediated agglutination and lectin-mediated substrate adhesion may be related. It is possible that this effect is similar to the binding of cells to Con A coated nylon fibres as described by Rutishauser and Edelman (1976).

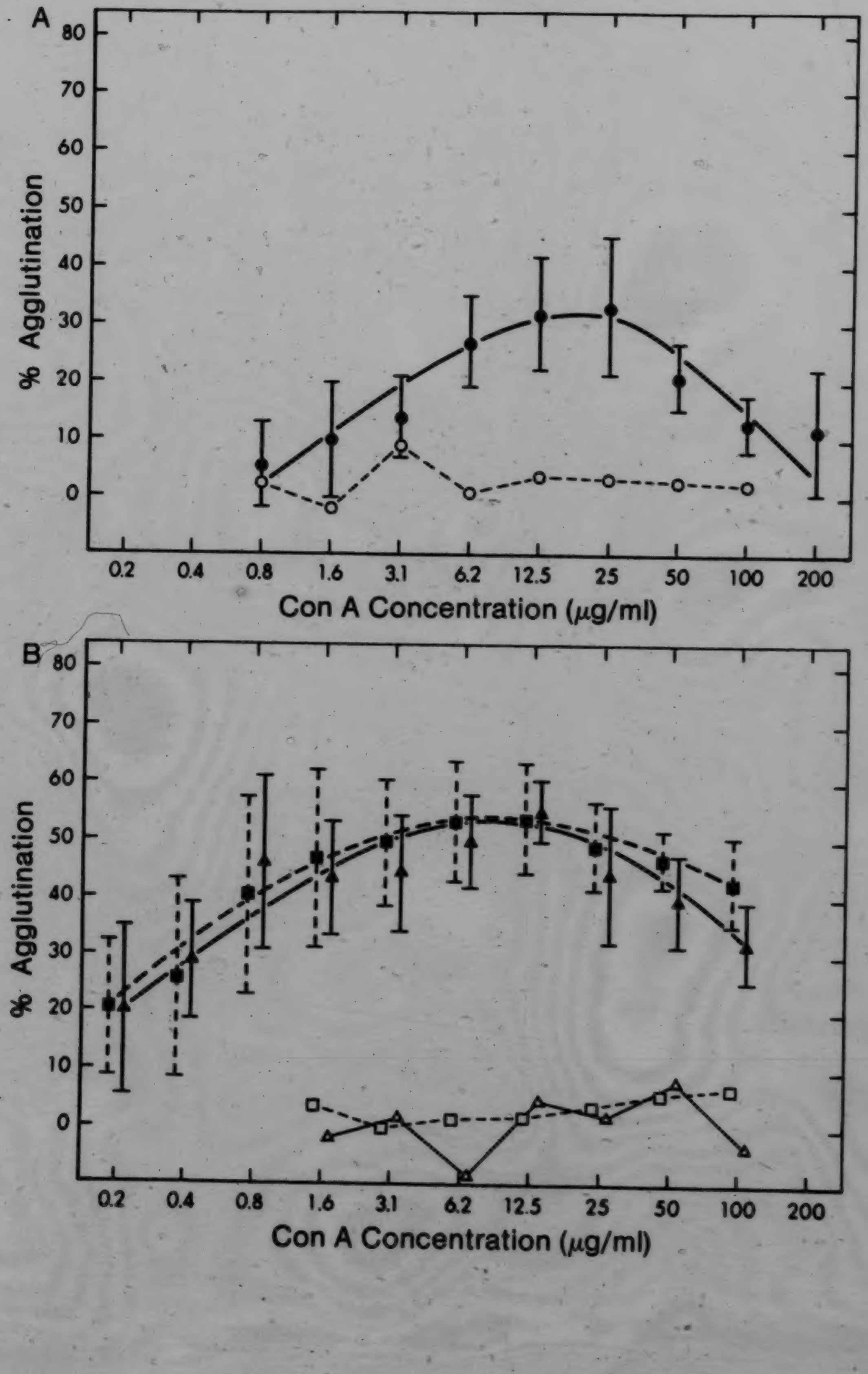
C. Dose Response of the Agglutination Reaction

Lectin dose response experiments were performed with Con A, WGA, RCA, and SBA on both AOEn and AP cells. For AP cells, the effect of the dissociation technique on lectin-mediated agglutinability was also examined. In all cases, the per cent agglutination was determined after a 20 minute incubation period at room temperature. In experiments where the effect of a hapten was investigated, the lectin and hapten were mixed together first before adding the cells.

Con A was the only lectin tested with which there was an appreciable difference in the agglutinability of AOEn and AP cells. AP cells were agglutinated by lower concentrations of Con A and to a greater extent than AOEn cells (Figures 11a and 11b). The Con A dose response curves of both cell types were interesting in that a "bell shaped" curve was obtained. There was a range of lectin concentrations which elicited a maximal response while higher and lower concentrations resulted in a submaximal response. AOEn cells were agglutinated maximally (approximately 30%) at Con A concentrations of 12.5 to 25 μg/ml (Figure 11a). Agglutination was less than 10% at Con A concentrations of less than 1.6 μg/ml and greater than 100 μg/ml. AP cells were agglutinated to a maximum of about 50% by 6.2 to 12.5 μg/ml Con A (Figure 11b). At 0.2 μg/ml Con A, AP agglutination was reduced to approximately 20%; 100 μg/ml Con A reduced agglutination to about 40%. There was essentially no difference in the agglutinability of EDTA or trypsin dissociated cells (Figure 11b). The hapten α-methyl-D-mannoside effectively reduced the aggultination of AOEn and AP cells dissociated by either method to less than 10% at all Con A concentrations tested (Figures 11a and 11b). It was observed that

Figure 11a. Con A dose response of area opaca endoderm cells. Approximately 1 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of Con A (-●-) or Con A + 50 mM α-methyl-D-mannoside (--o--). The per cent agglutination was determined after 20 minutes. Each point in the curve of Con A alone is the average of 4 to 9 replicates. The bars represent ± 1 standard deviation. The curve of Con A + hapten is from one experiment.

Figure 11b. Con A dose response of area pellucida cells. Area pellucidae were dissociated with either trypsin (squares) or EDTA (triangles). Approximately 1.2 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of Con A (closed symbols) or Con A + 25 mM α-methyl-D-mannoside (open symbols). The per cent agglutination was determined after 20 minutes. Each point in the curves of Con A alone is the average of 4 to 7 replicates. The bars represent ± 1 standard deviation. The curves of Con A + hapten are from one experiment. The points in the graph are staggered at each lectin concentration for clarity.


A
80
70
60
50
40
30
20
10
0
% Agglutination
0.2
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
Con A Concentration (μg/ml)
B
80
70
60
50
40
30
20
10
0
% Agglutination
0.2
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
Con A Concentration (μg/ml)

this hapten, as well as the haptens for the other lectins, occasionally reduced agglutination to less than 0%, i.e., after the 20 minute incubation there were more single cells present in the sample containing lectin and hapten than in the lectin free control. This may have been due to slight variations in the initial inoculations and/or fluctuations in the base line amount of spontaneous reaggregation which occurred in each sample.

Succinylation of Con A has been reported to cause the dissociation of the native, tetrameric, form of the lectin into a dimeric state without affecting its carbohydrate binding specificity (Gunther et al., 1973). The tetrameric form, however, appears to be necessary to mediate an agglutination response. The inability of the succinylated Con A preparation to cause agglutination is illustrated in Figure 12. Lectin concentrations ranging from 0.8 to 200 μg/ml did not agglutinate AOEn cells.

WGA, RCA, and SBA elicited more typical dose responses compared to Con A; the dose curves obtained for the former three lectins was more sigmoidal in shape. Both AOEn and AP cells exhibited similar dose reponses to WGA (Figures 13a and 13b). Agglutination of AOEn, trypsin dissociated AP, and EDTA dissociated AP cells exceeded 10% at WGA concentrations greater than 3 μg/ml and reached a maximum of approximately 50% agglutination at 25 μg/ml. Further increases of the WGA concentration did not appreciably alter the extent of agglutination. The inclusion of 50 mM GlcNac did not inhibit WGA-mediated agglutination completely, particularily at

Figure 12. Succinylated Con A dose response of area opaca endoderm cells. Approximately 1 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of succinylated Con A. The per cent agglutination was determined after 20 minutes. Each point is the average of 2 replicates. The bars represent ± 1 standard deviation.

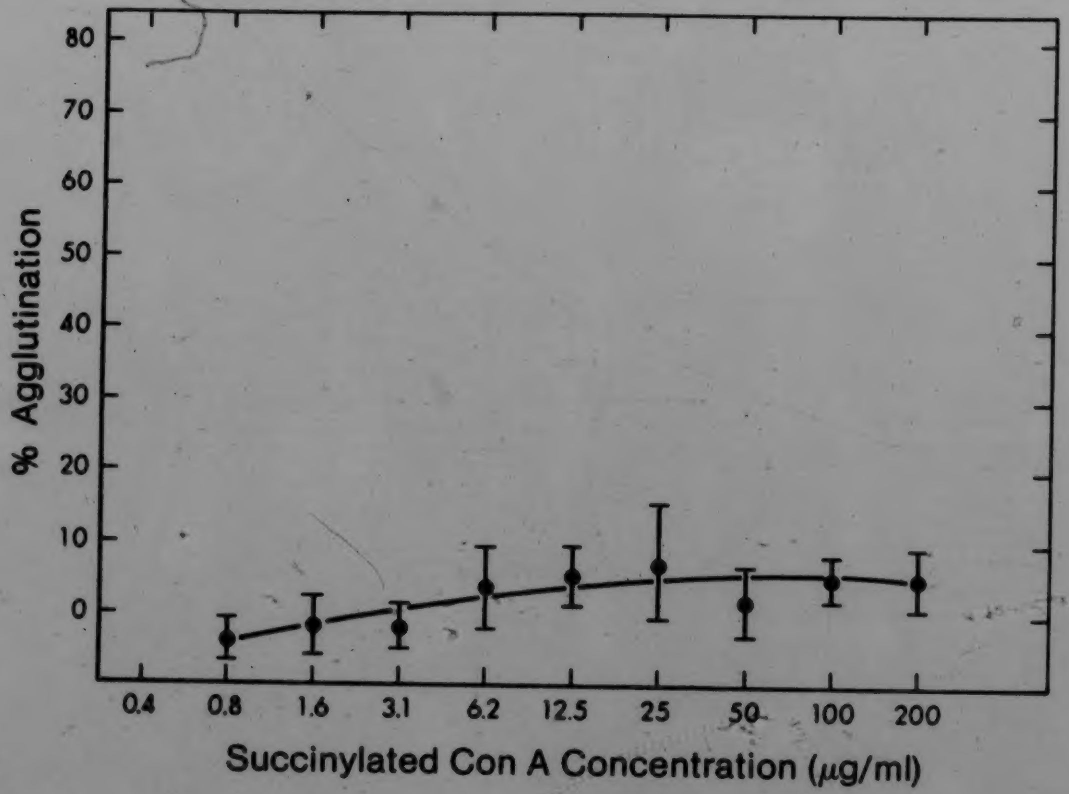
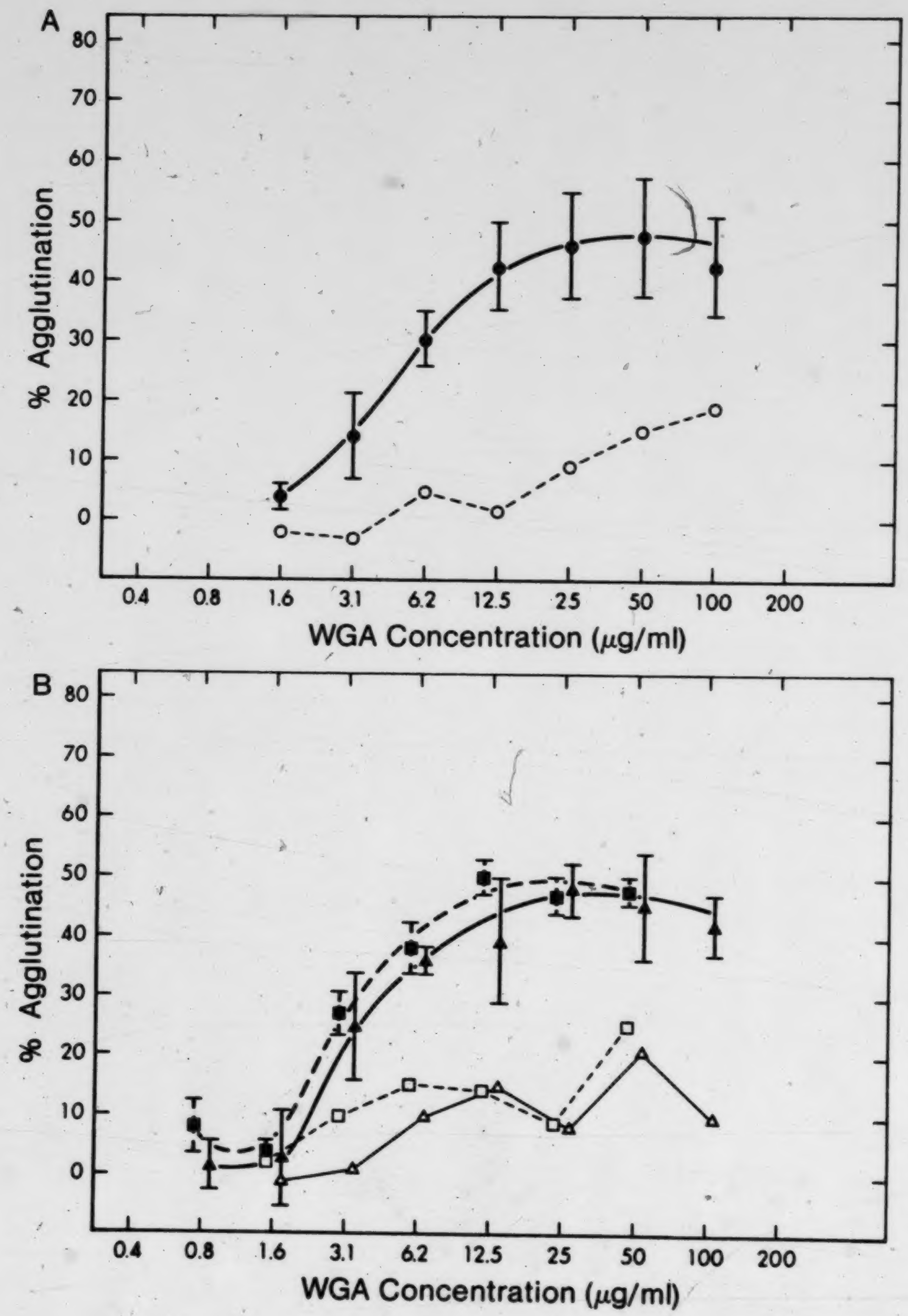

% Agglutination
80
70
60
50
40
30
20
10
0
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
Succinylated Con A Concentration (μg/ml)

Figure 13a. WGA dose response of area opaca endoderm cells. Approximately 1 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of WGA (-●-) or WGA + 50 mM GlcNac (--o--). The per cent agglutination was determined after 20 minutes. Each point in the curve of WGA alone is the average of 5 or 6 replicates. The bars represent ± 1 standard deviation. The curve of WGA + hapten is the average of duplicate determinations.

Figure 13b. WGA dose response of area pellucida cells. Area pellucidae were dissociated with either trypsin (squares) or EDTA (triangles). Approximately 1.2 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of WGA (closed symbols) or WGA + 50 mM Glc Nac (open symbols). The per cent agglutination was determined after 20 minutes. Each point in the curves of WGA alone is the average of 3 replicates. The bars represent ± 1 standard deviation. The curves of WGA + hapten are from one experiment. The points in the graph are staggered at each lectin concentration for clarity.


A
80
70
60
50
40
30
20
10
0
% Agglutination
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
WGA Concentration (μg/ml)
B
80
70
60
50
40
30
20
10
0
% Agglutination
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
WGA Concentration (μg/ml)

the higher lectin concentrations (Figures 13a and 13b).

The greatest agglutination response was elicited by RCA. Both AOEn and AP cells exhibited a maximal agglutination of about 65% at lectin concentrations greater than 12.5 μg/ml (Figures 14a and 14b). There was essentially no difference in the agglutinability of AOEn, trypsin dissociated AP, and EDTA dissociated AP cells. The hapten β-lactose was a very effective inhibitor of RCA-mediated agglutination. One experiment, illustrated in Figure 14a, shows the reduction of RCA-mediated agglutination of AOEn cells to less than 5% by 50 mM β-lactose. Similarily, 25 mM β-lactose reduced the RCA-mediated agglutination of AP cells, dissociated by either method, to less than 10% (Figure 14b). As β-lactose and thiodigalactoside are the two most potent inhibitors of the endogenous chick lectin described by Cook et al. (1979), the ability of TDG to act as a haptenic inhibitor for RCA was examined. Both β-lactose and TDG caused a 50% reduction of RCA-mediated agglutination of AOEn cells at a concentration of 0.2 mM (Figure 15). Agglutination was reduced to less than 10% at hapten concentrations greater than 1 mM.

The effect of neuraminidase treatment on SBA-mediated agglutination is shown in Figures 16a and 16b. As mentioned previously, SBA did not agglutinate cells which had not been pretreated with NaNase. Agglutination of AOEn cells was already evident at 12.5 μg/ml of this lectin and reached a maximum of 55% at 200 μg/ml SBA (Figure 16a). Agglutination was reduced to less than

Figure 14a. RCA dose response of area opaca endoderm cells. Approximately 1 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of RCA (-●-) or RCA + 50 mM β-lactose (--o--). The per cent agglutination was determined after 20 minutes. Each point in the curve of RCA alone is the average of 3 to 8 replicates. The bars represent ± 1 standard deviation. The curve of RCA + hapten is the average of duplicate determination.

Figure 14b. RCA dose response of area pellucida cells. Area pellucidae were dissociated with either trypsin (squares) or EDTA (triangles). Approximately 1.2 x 10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of RCA (closed symbols) or RCA + 25 mM β-lactose (open symbols). The per cent agglutination was determined after 20 minutes. Each point in the curves of RCA alone is the average of 3 to 7 replicates. The bars represent ± 1 standard deviation. The curves of RCA + hapten are the averages of duplicate determination. The points in the graph are staggered at each lectin concentration for clarity.

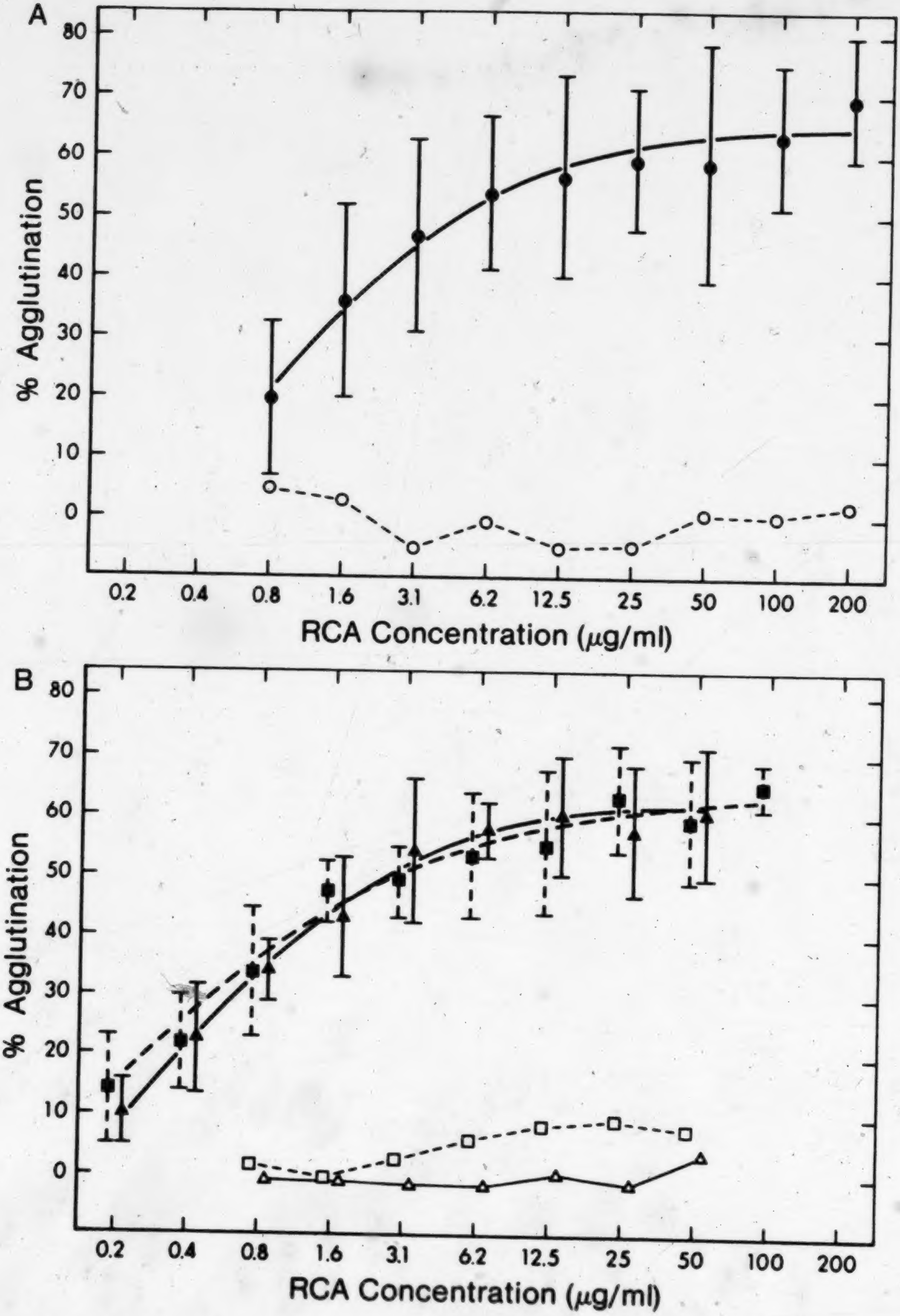
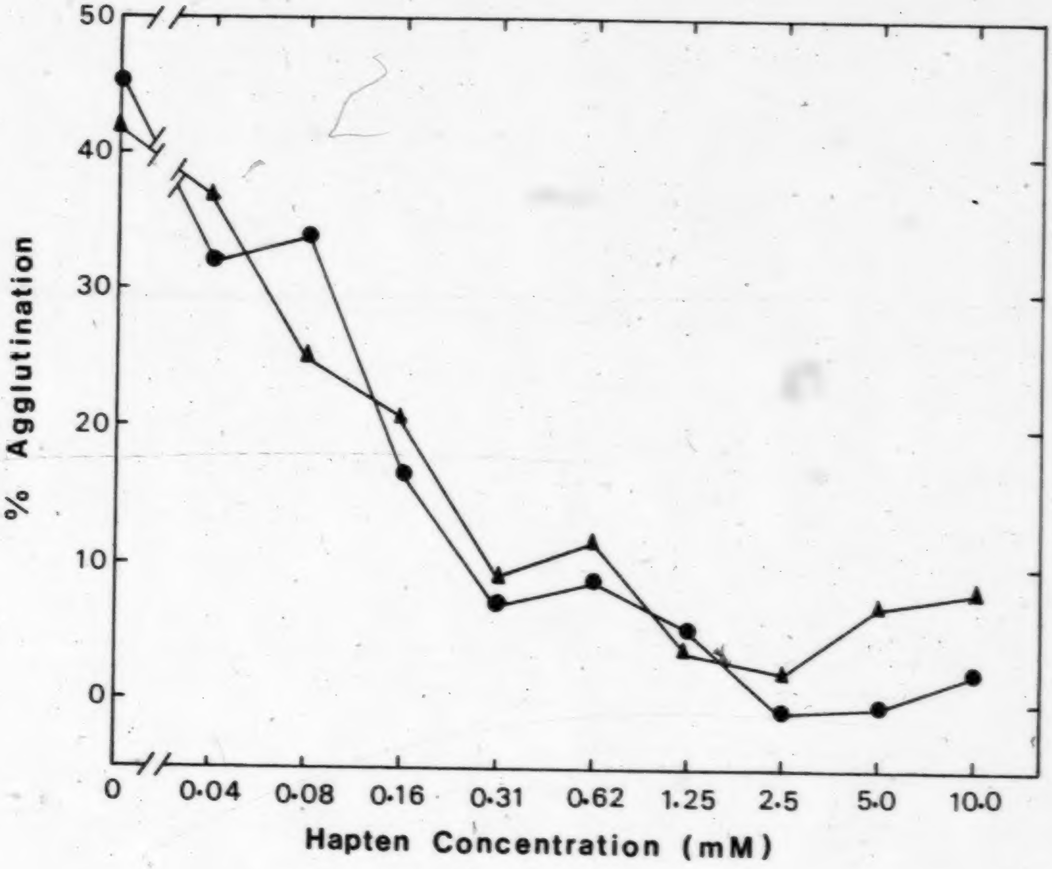

A
80
70
60
50
40
30
20
10
0
% Agglutination
0.2
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
RCA Concentration (μg/ml)
B
80
70
60
50
40
30
20
10
0
% Agglutination
0.2
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
200
RCA Concentration (μg/ml)

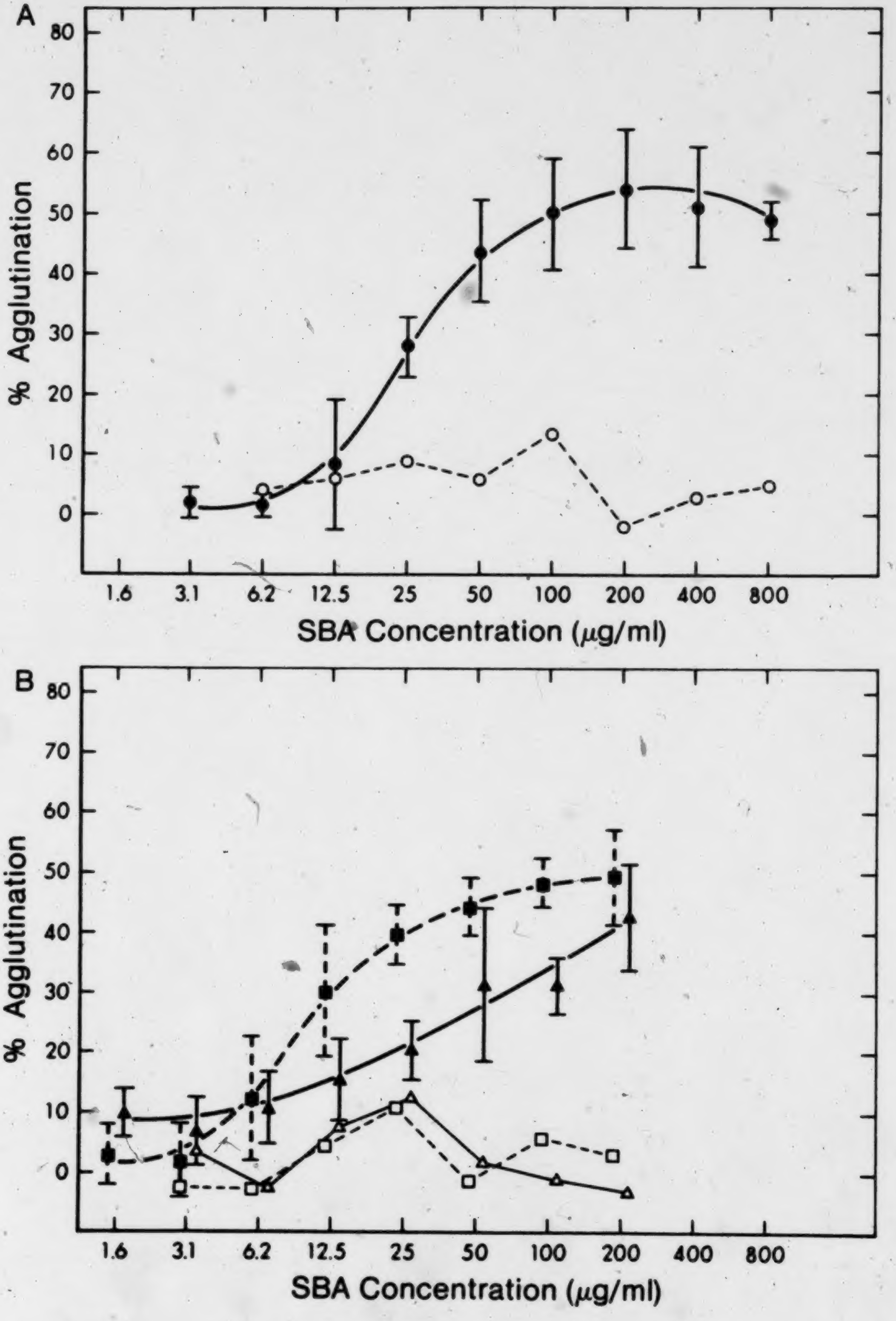
Figure 15. Haptenic inhibition of RCA-mediated agglutination of area opaca endoderm cells. Approximately 1 x 10^4 cells were rotated at 135 rpm at room temperature in the presence of 10 μg/ml RCA and varying concentrations of β-lactose (circles) or thiodigalactoside (triangles). The per cent agglutination was determined after 20 minutes. Each point is the average of duplicate determinations.



50
40
30
20
10
0
% Agglutination
0
0·04
0·08
0·16
0·31
0·62
1·25
2·5
5·0
10·0
Hapten Concentration (mM)

Figure 16a. SBA dose response of neuraminidase treated area opaca endoderm cells. Approximately 1×10^4 cells were rotated at 135 rpm at room temperature in the presence of SBA (-●-) or SBA + 50 mM GalNac (--o--). The per cent agglutination was determined after 20 minutes. Each point in the curve of SBA alone is the average of 3 to 10 replicates. The bars represent ± 1 standard deviation. The curve of SBA + hapten is the average of duplicate determinations.

Figure 16b. SBA dose response of neuraminidase treated area pellucida cells. Area pellucidae were dissociated with either trypsin (squares) or EDTA (triangles). Approximately 1.2×10^4 cells suspended in PCS were rotated at 135 rpm at room temperature in the presence of SBA (closed symbols) or SBA + 35 mM GalNac (open symbols). The per cent agglutination was determined after 20 minutes. Each point in the curves of SBA alone is the average of 3 or 4 replicates. The bars represent ± 1 standard deviation. The curves of SBA + hapten are from one experiment. The points in the graph are staggered at each lectin concentration for clarity.


A
B
% Agglutination
80
70
60
50
40
30
20
10
0
1.6
3.1
6.2
12.5
25
50
100
200
400
800
SBA Concentration (μg/ml)

15% by 50 mM GalNac (Figure 16a). A comparable extent of SBA-mediated agglutination was observed for AP and AOEn cells, but the dissociation method appeared to affect AP cell agglutination with this lectin. At intermediate concentrations of SBA (12.5 to 100 μg/ml) trypsin dissociated AP cells were agglutinated to a greater extent than EDTA dissociated cells (Figure 16b). However, only at 25 and 100 μg/ml SBA was the difference between the two dissociations found to be statistically significant ($P<0.01$ by unpaired T-test). The maximum agglutination attained was similar for cells obtained by both dissociations (approximately 45% at 200 μg/ml SBA) The hapten GlcNac, at a concentration of 35 mM, inhibited the agglutination of AP cells dissociated by either method.

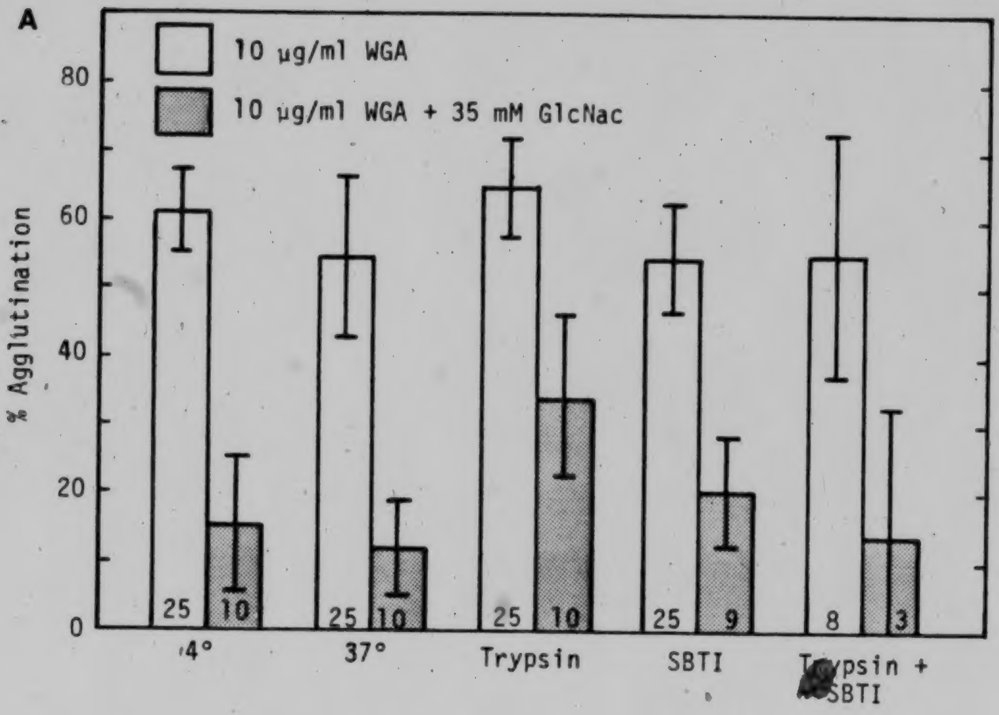
D. Trypsin Treatment of Area Opaca Endoderm Cells

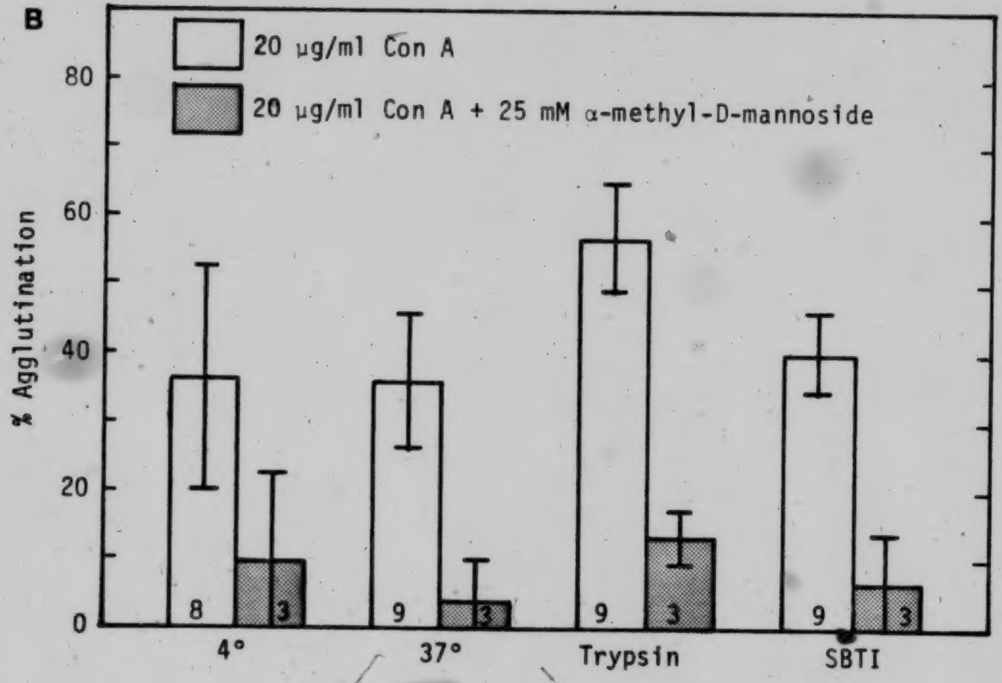
Endoderm cells of the area opaca can be dissociated by fairly mild methods involving only the removal of calcium and magnesium. It was important to determine if subsequent trypsinization of dissociated AOEn cells affected their lectin-mediated agglutinability. For this purpose, experiments were designed in which freshly dissociated AOEn cells were incubated at 37°C in the presence of trypsin, trypsin with SBTI, or SBTI alone, as described in the Materials and Methods. The latter case, SBTI alone, was performed in order to inhibit any possible endogenous trypsin like protease which may be present in these cells. Two untreated control incubations were also performed; one cell suspension was incubated at 37^{o}C and the other was incubated on ice. Following the incubation, the suspensions were washed with PCS, resuspended in PCS at equal cell concentrations and assayed for their lectin-mediated agglutinability. The cell suspensions were maintained on ice for the duration of the experiment.

The effect of the above mentioned treatments on WGA-mediated agglutination is illustrated in Figure 17a. The untreated sample which had been incubated on ice (4^{o}C control) was agglutinated to about 60% by 10 μg/ml WGA. The presence of 35 mM GlcNac reduced the agglutination of these cells to approximately 15%. The 4^{o}C control incubation was performed as this most closely represented the situation in the other experiments in this investigation, for example in the lectin dose response experiments. The 37^{o}C control incubation, however, is a better comparison for the other treatments in this series of experiments. This is made apparent in Figure 17a where it can be seen that cells incubated at 37^{o}C were agglutinated to a lesser extent than those kept at 4^{o}C. This difference, of approximately 6%, was determined to be significant by an unpaired T-test ($P<0.05$). Treatment with 50 units trypsin per area opaca significantly increased the WGA-mediated agglutinability by approximately 10% over the 37^{o}C control ($P<0.01$); interestingly there was no significant difference between trypsin treated cells and the 4^{o}C control cells. The hapten GlcNac was not as effective as an inhibitor of agglutination of the trypsin treated cells; 35 mM GlcNac only reduced the agglutination to 35%

Figure 17a. Effect of trypsinization of WGA-mediated agglutination of area opaca endoderm cells. Freshly dissociated cells were incubated for 20 minutes on ice ($4^{o}C$), or at $37^{o}C$ either alone, in the presence of 50 units trypsin/area opaca, in the presence of soybean trypsin inhibitor (SBTI) at a sufficient concentration to inhibit the amount of trypsin in the previous case, or in the presence of trypsin plus SBTI at the same concentrations as in the preceeding cases. Following the incubation the cells were washed and assayed for their agglutinability with 10 μg/ml WGA or 10 μg/ml WGA + 35 mM GlcNac. The average values of repicate agglutination assays are plotted. The numbers at the bottom of the graph refer to the number of replicates. The bars represent ± 1 standard deviation.

Figure 17b. Effect of trypsinization on Con A-mediated agglutination of area opaca endoderm cells. Cells were treated similarily as described in Figure 17a and assayed for their agglutinability with 20 μg/ml Con A or 20 μg/ml Con A + 25 mM α-methyl-D-mannoside. The average values of replicate agglutination assays are plotted. The numbers at the bottom of the graph refer to the number of replicates. The bars represent ± 1 standard deviation.


A
10 μg/ml WGA
10 μg/ml WGA + 35 mM GlcNac
% Agglutination
0
20
40
60
80
25
10
25
10
25
10
25
9
8
3
4°
37°
Trypsin
SBTI
Trypsin + SBTI

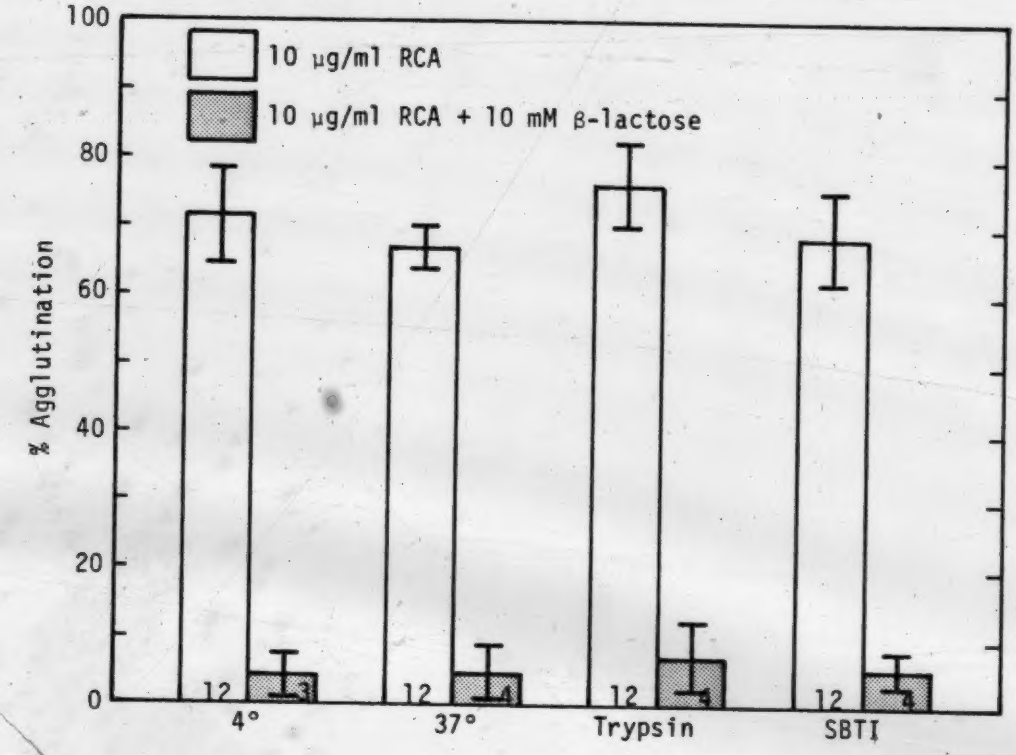

B
20 μg/ml Con A
20 μg/ml Con A + 25 mM α-methyl-D-mannoside
% Agglutination
0
20
40
60
80
8
3
9
3
9
3
9
3
4°
37°
Trypsin
SBTI

as opposed to 15 to 20% for the other samples. This was a significant difference from the 4°C and 37°C controls with hapten ($P<0.01$) and from the SBTI treatment with hapten ($P<0.05$). The presence of SBTI alone did not alter the agglutinability appreciably from that observed for the 37°C control. The agglutinability of cells incubated with SBTI and trypsin was similar to the 37°C control and the SBTI treated cells. The trypsin plus SBTI sample did not differ significantly from any of the other samples, however, there was a lower sample size and hence a greater standard deviation in this case.

Con A-mediated agglutinability of AOEn cells was also increased after trypsin treatment (Figure 17b). Trypsin treated cells were approximately 20% more agglutinable than the 4°C control, 37°C control and the SBTI treated cells. Analysis of the data by an F-test and Duncan's multiple range test, revealed that this was a significant difference ($P<0.01$). There was no significant difference between the agglutinability of the 4°C and 37°C controls, and the SBTI treated sample. The hapten α-methyl-D-mannoside reduced the agglutination to less than 10% except in the case of the trypsin treated cells where it reduced it to about 14%, however, this difference was not found to be significant.

Trypsin treatment significantly increased the RCA-mediated agglutinability of AOEn cells by about 10% over the 37°C control and SBTI treatment ($P<0.01$ and $P<0.05$ respectively) (Figure 18). The case with RCA was similar to that for WGA in that the trypsin

Figure 18. Effect of trypsinization on RCA-mediated agglutination of area opaca endoderm cells. Cells were treated similarily as described in Figure 17a and assayed for their agglutinability with 10 μg/ml RCA or 10 μg/ml RCA + 10 mM β-lactose. The average values of replicate agglutination assays are plotted. The numbers at the bottom of the graph refer to the number of replicates. The bars represent ± 1 standard deviation.


10 μg/ml RCA
10 μg/ml RCA + 10 mM β-lactose
% Agglutination
100
80
60
40
20
0
12
3
12
4
12
4
12
4
4°
37°
Trypsin
SBTI

treatment at 37°C did not significantly alter the agglutinability from the non-enzyme treated 4°C control. Incubation at 37°C slightly reduced the agglutinability of the control cells compared to those incubated at 4°C, but this difference was not significant. The agglutination of all four samples was reduced to less than 10% by the presence of 10 mM β-lactose.

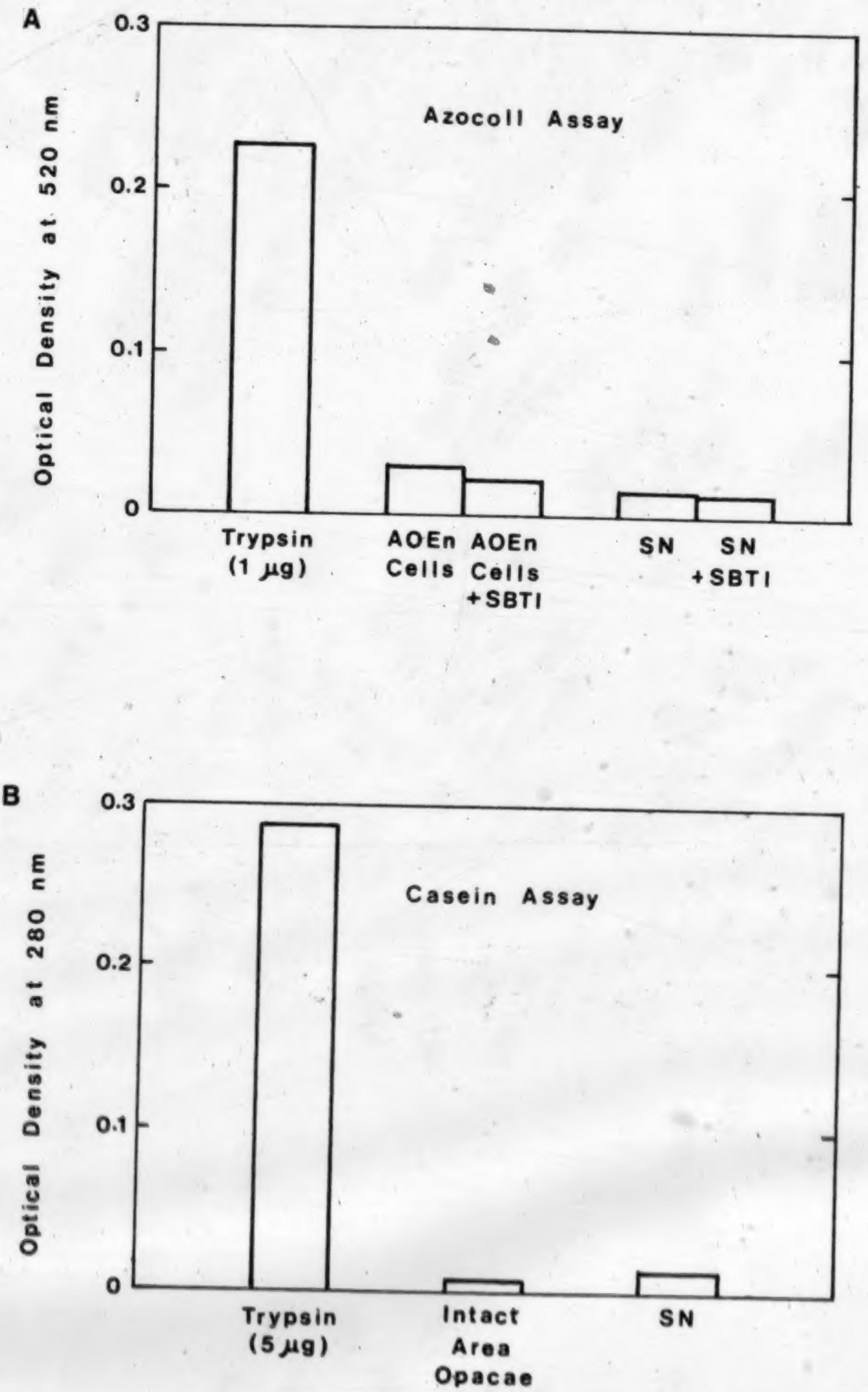
It should be mentioned that all of the differences of agglutinability mentioned in the preceeding paragraphs could be detected reliably with the Coulter Counter. The visual assessment of agglutination, however, was not discriminating enough to consistently detect these differences accurately.

E. Assay for Endogenous Protease Activity

As the *in vivo* fate of the AOEn cells is to form the absorptive layer of the yolk sac (Romanoff, 1960), it was reasoned that high levels of hydrolytic enzymes, including proteases, may be present in suspensions of dissociated cells. Two different enzyme assays, however, failed to detect appreciable levels of endogenous protease activity under similar experimental conditions as in the previous series of experiments. Each assay was performed at least three times; representative experiments are illustrated in Figures 19a and 19b. The enzymatic activity of 1 μg trypsin (12 units) was considerably greater than an AOEn cell suspension (4×10^5 cells), with or without 50 μg SBTI, and 1 ml of a cell free supernatant, with or without 50 μg SBTI, as determined by an Azocoll

Figure 19a. Azocoll protease assay of area opaca endoderm cells (AOEn) and cell free supernatants (SN) prepared from dissociations of AOEn cells. Assays were performed in a total volume of 5 ml PCS with 25 mg Azocoll. Samples were incubated 15 minutes at 37°C and the reaction was terminated by filtration. The optical density of the filtrate was read at 520 nm. Approximately 4 x 10^5 cells in the absence or presence of 50 μg soybean trypsin inhibitor (SBTI) and 1 ml SN in the absence or presence of 50 μg SBTI had considerably less demonstratable protease activity than 1 μg trypsin (12 units).

Figure 19b. Casein digestion protease assay of intact area opacae and cell free supernatant (SN) prepared from dissociations of area opaca endoderm cells. Fifteen intact area opacae or 1 ml SN were incubated with 0.5% (w/v) casein in a total volume of 2 ml for 60 minutes in the case of area opacae or 20 minutes in the case of SN. The reaction was terminated by adding 3 ml 5% (w/v) trichloroacetic acid and the optical density of the supernatant read at 280 nm. Trypsin (5 μg or 60 units) was incubated for 20 minutes and assayed similarily.


A
Azocoll Assay
Optical Density at 520 nm
0.3
0.2
0.1
0
Trypsin (1 µg)
AOEn Cells
AOEn Cells +SBTI
SN
SN +SBTI
B
Casein Assay
Optical Density at 280 nm
0.3
0.2
0.1
0
Trypsin (5 µg)
Intact Area Opacae
SN

assay (Figure 19a). The supernatant was obtained by pooling supernatant 1 and 2 (see Figure 2) from an AOEn cell dissociation and centrifuging at high speed to pellet the cells. The cells and the supernatant both consistently had a slight increase in the optical density, however, the results for the SBTI treated samples were more variable. The presence of SBTI decreased this change in optical density a very small extent. In the same experiment as illustrated in Figure 19a, 20 μg SBTI completely inhibited the activity of 20 μg trypsin.

The results of a casein digestion assay are shown in Figure 19b. Fifteen intact area opacae and 1 ml cell free supernatant induced a very small increase in optical density at 280 nm when compared to that induced by 5 μg trypsin.

It should be mentioned that in the preceeding series of experiments where the effect of trypsin on agglutination was examined, the concentration of trypsin was very high compared to the endogenous levels of protease detected. In a typical experiment where the cells dissociated from 20 area opacae were treated, the final trypsin concentration, at 50 units per area opaca, would have been 1000 units, or 80 μg per ml. Any endogenous protease activity, therefore, would have been insignificant compared to the large amounts of trypsin added.

F. Aggregation of Blastoderm Cells

Cells dissociated from whole blastoderms were cultured in L-15 medium in rotating flasks at 37°C for 20 to 24 hours; subsequently, the medium was supplemented with 15% (v/v) FCS and the cells were incubated further for up to three days. Under these experimental conditions the cells rapidly reaggregated and eventually underwent cell sorting and limited differentiation. Blastoderms were dissociated with either EDTA or trypsin but the dissociation technique did not appear to affect either the aggregation or differentiation, thus, the results will be discussed collectively.

At 34 hours of incubation of the aggregates, evidence of cell sorting was apparent as the cells were organized into two distinct phases which were characterized by their degree of compactness (Figure 20). This pattern of cell sorting has been described previously (Zalik and Sanders, 1974; Sanders and Zalik, 1976) in aggregates formed by cells from unincubated to head process stage chick embryos. These authors surmised that the more compact phase was formed by cells derived from the epiblast and area pellucida. The compact phase was usually completely invested by loosely packed cells which were determined to be derived from the hypoblast by Zalik and Sanders (1974) and Eyal-Giladi et al. (1975).

The presence of 0.5 μg/ml Con A or 1 μg/ml WGA during the first 24 hours of incubation did not appear to affect the development of the aggregates appreciably, as observed at 34 hours (Figures 21 and 22). Both the extent and pattern of cell sorting

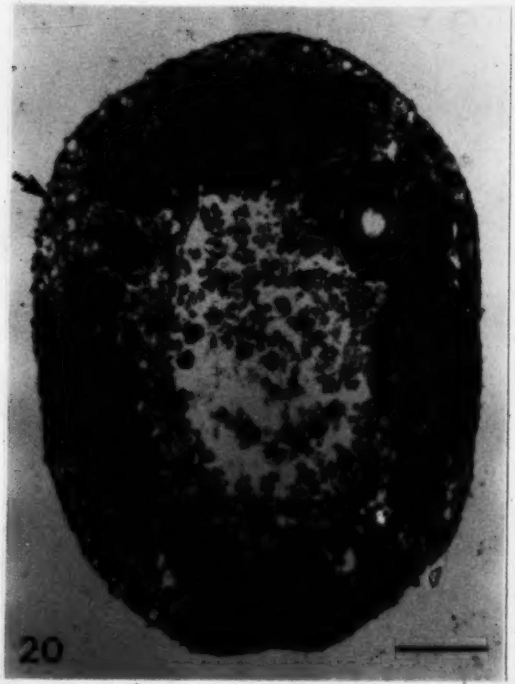
Figures 20-23. Aggregates of trypsin dissociated blastoderm cells. Cells were cultured for 34 or 46 hours, fixed in Bouin's, sectioned in paraffin at 7 μm and stained with Haematoxylin and Eosin. The bar is 100 μm.

Figure 20. A control aggregate (34 h) showing the initial sorting out into compact (*) and loose (arrow) cell phases. Note the presence of the central cavity which contains some cells, cell debris, and yolk spheres.

Figure 21. A 34 hour aggregate which had been formed in the presence of 0.5 μg/ml Con A for the first 24 hours of culture. There is evidence of cell sorting into loose (arrow) and compact (*) phases.

Figure 22. A noncavitated 34 hour aggregate which had been formed in the presence of 1 μg/ml WGA during the first 24 hours of culture. The cell sorting is similar to that in Figures 20 and 21.

Figure 23. A control aggregate (46 h). Numerous small cavities are present within the compact phase.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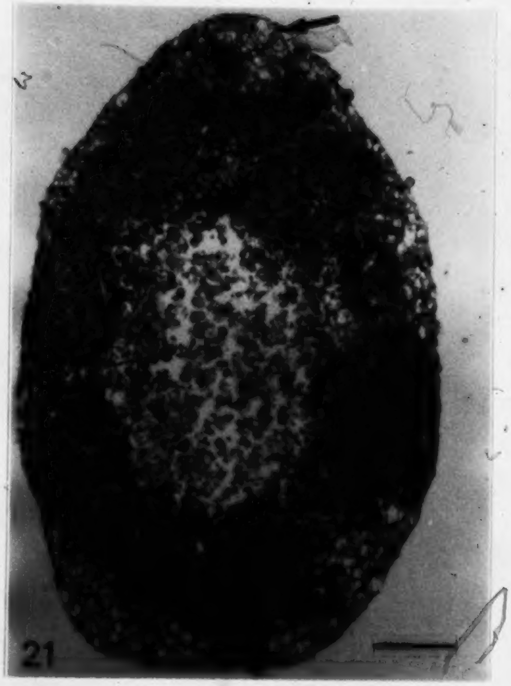
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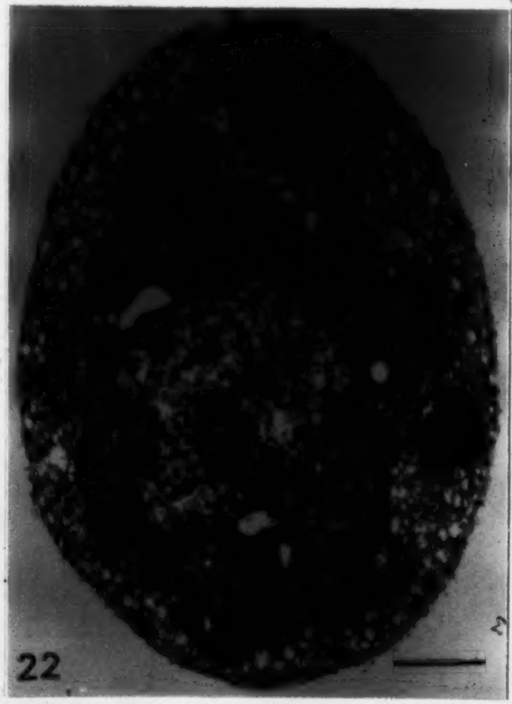
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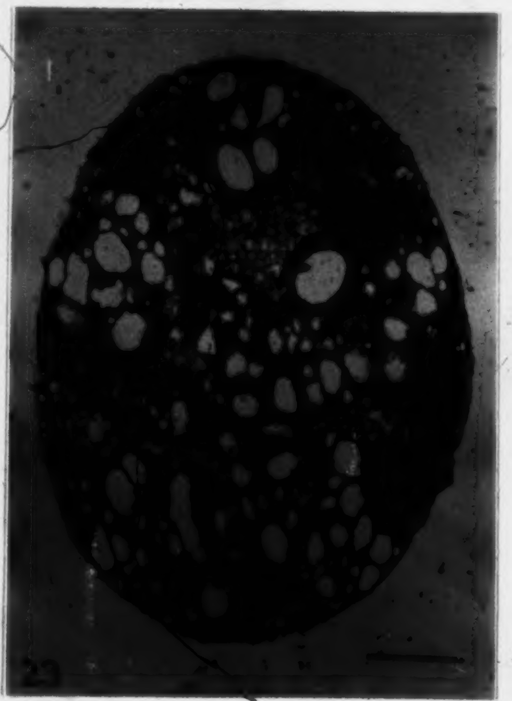
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was similar to that observed in the control aggregates. The haptens for these lectins, α-methyl-D-mannoside and GlcNac (50 mM), also had no effect on the aggregation when present during the first 24 hours of incubation either alone, or in combination with their respective lectin.

A predominant feature of a large proportion of the 34 hour aggregates was the presence of a large central cavity (Figures 20 and 21). The cavity often contained yolk spheres and what appeared to be cellular debris. The presence of a cavity became less common in aggregates which had been cultured for longer periods of time. Often, the center of older aggregates was characterized by numerous small, irregular cavities (Figure 23). Whether this was due to a collapse of the cavity or an active proliferation and migration of cells into the space is uncertain. This phenomenon has also been observed in aggregates of isolated AOEn cells (Milos et al., 1979a).

After 96 hours in culture the most consistant feature of the control aggregates was the division of the aggregate into two distinct regions (Figure 24). The cells in the peripheral region were fairly loosely packed and highly vacuolated. Sanders and Zalik (1976) likened these external sorting cells to yolk sac endoderm due to their similar appearance (Figure 25). The yolk sac-like cells were usually bounded internally by a layer of dense connective tissue consisting of elongated cells (Figure 26). Internal to this layer the cells were notably different in structure

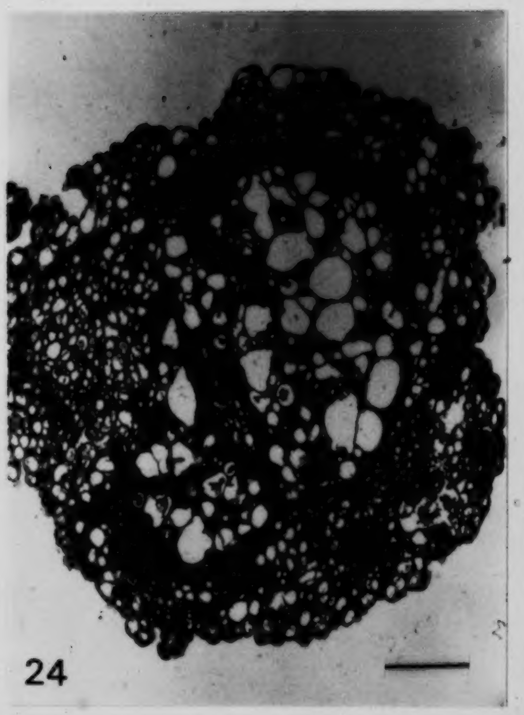
Figure 24. A 96 hour aggregate of EDTA dissociated blastoderm cells showing the yolk sac endoderm-like cells at the periphery. The center of the aggregate has a trabecular appearance. A layer of connective tissue separate the external and internal phases. The bar is 100 μm.

Figure 25. A section of the yolk sac of a 4½ day chick embryo. The embryo was fixed, sectioned, and stained as described for Figures 20 to 23. The endoderm cells (En) are characterized by the large vacuoles. The bar is 25 μm.

Figure 26. A section of a 96 hour aggregate of EDTA dissociated blastoderm cells showing the layer of connective tissue separating the yolk sac endoderm-like cells on the right from the trabecular network of cells on the left. The cavities in the inner area of the aggregate are often bounded by thinned cells or cell processes (arrow). The bar is 25 μm.

Figure 27. An aggregate (96 h) which shows differentiation of formed blood elements (B) and the presence of a differentiated tubule. The bar is 100 μm.

Figure 28. A section through the mesonephros of a 4½ day chick embryo. The bar is 25 μm.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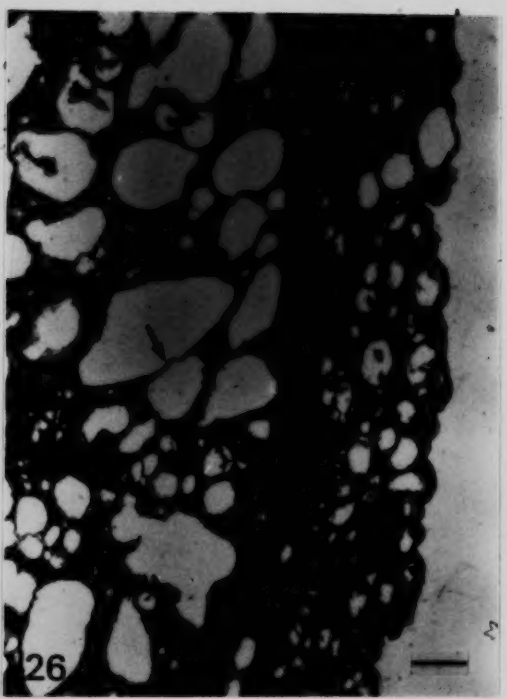
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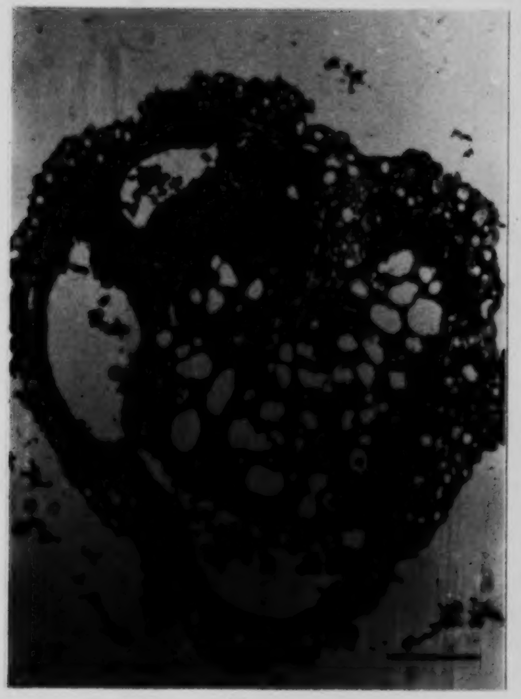

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and organization from the cells at the periphery. The internal structure of the aggregate was trabecular in appearance; it was characterized by numerous small cavities which were bounded by cells and thinned cell processes (Figure 26). Within the central portion of the aggregate there were often islands of more densely packed cells. In a few instances well differentiated tubules were present (Figure 27). The columnar cells that bounded the lumen of the tubules were possibly of mesodermal origin as they had a very similar appearance to the cells in the mesonephric tubules of a four and a half day chick embryo (Figure 28). Formed blood elements were often present within the aggregates; these were present in both the external and internal regions. The blood elements were either densely packed within the surrounding tissue or, more commonly, they were present within large cavities which appeared to be lined by an endothelium (Figure 27).

The presence of WGA, Con A, or succinylated Con A during the initial 24 hour culture period did not appear to alter the aggregative behaviour of the cells as observed at 96 hours. WGA was tested at 0.1, 0.5, and 1.0 μg/ml. Con A and succinylated Con A were tested at the above concentrations as well as at 5 and 10 μg/ml. The effect of 10 and 25 mM α-methyl-D-mannoside, either alone, or in combination with Con A or succinylated Con A was also tested. In all of the above situations the appearance of 96 hour aggregates was grossly similar to the control aggregates (Figures 29, 30, and 31). Lectin treatment, however, appeared to have a

Figures 29 to 34. Lectin treated aggregates (96 h) of EDTA dissociated blastoderm cells. The lectin was present during the first 24 hours, subsequent culturing was in lectin free medium. Stained with Haematoxylin and Eosin.

Figure 29. An aggregate formed in the presence of 1.0 μg/ml WGA. The bar is 100 μm.

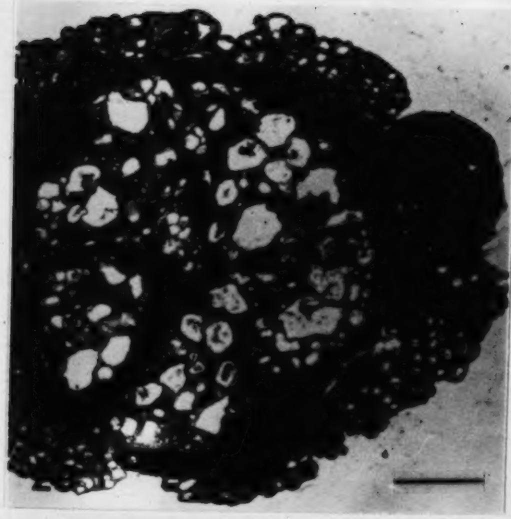
Figure 30. An aggregate formed in the presence of 1.0 μg/ml Con A. The bar is 100 μm.

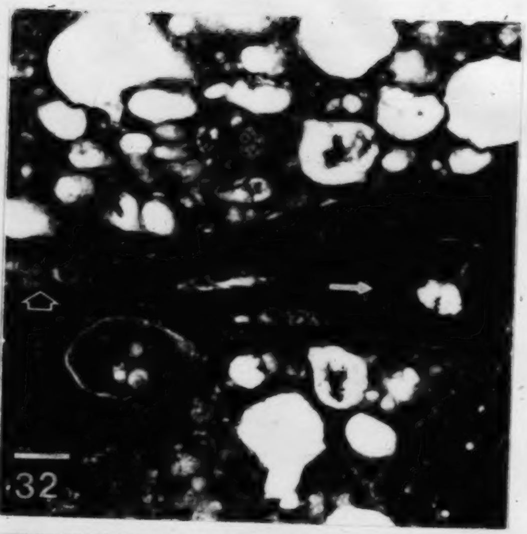
Figure 31. An aggregate formed in the presence of 0.5 μg/ml succinylated Con A. The bar is 100 μm.

Figure 32. A region of an aggregate formed in the presence of 0.5 μg/ml WGA showing the presence of a tubule (solid arrow) and some blood elements (open arrow). The bar is 25 μm.

Figure 33. A region of an aggregate formed in the presence of 5 μg/ml Con A showing an incompletely formed tubule (arrow) and some blood elements present within a cavity (B). The bar is 50 μm.

Figure 34. A region of an aggregate formed in the presence of 5 μg/ml succinylated Con A showing some tightly packed blood elements (arrow). The bar is 50 μm.

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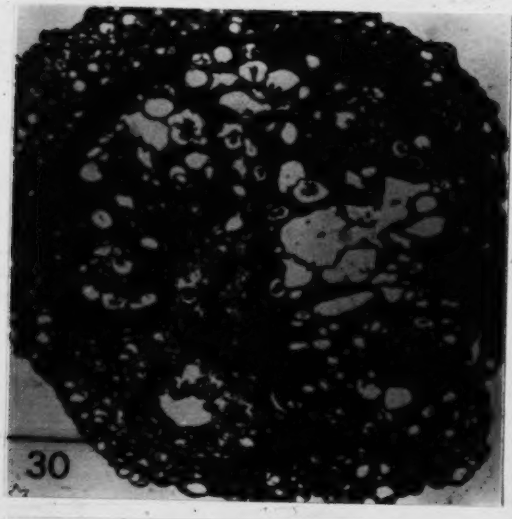
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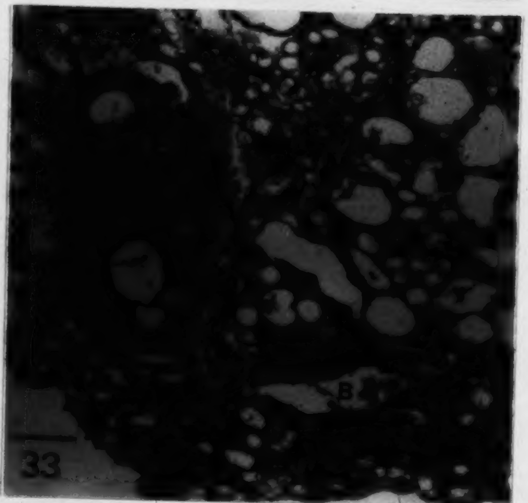
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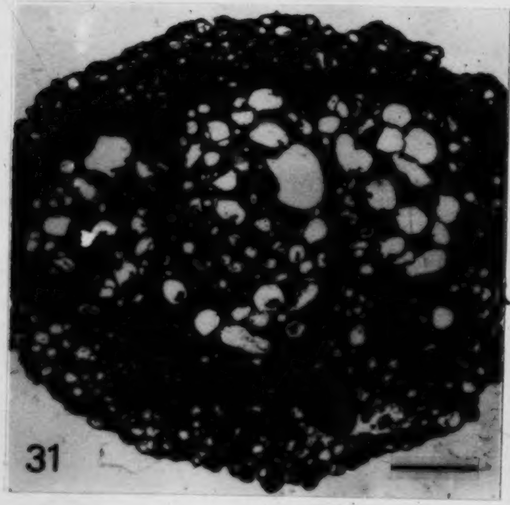
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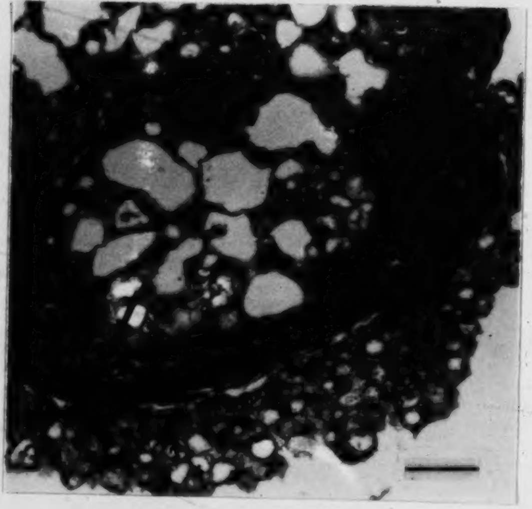

slight effect on the extent of differentiation achieved by the aggregates. Aggregates which had been formed in the presence of WGA were essentially similar to the controls in terms of the differentiated states observed. Blood elements were present in the majority of WGA treated aggregates, and in one case, a tubule, similar to those present in the controls was observed (Figure 32). Succinylated Con A and native Con A treated aggregates were never as highly organized as the most differentiated control aggregates, such as that shown in Figure 27. However, the treated aggregates were usually similar to the less differentiated controls such as that shown in Figure 24. Formed blood elements were common in succinylated Con A treated aggregates (Figure 34) but were rarely observed in Con A treated aggregates. In one Con A treated aggregate that was examined there was what appeared to be an attempt by mesodermal cells to organize into a tubular structure (Figure 33). This particular aggregate was one of the few Con A treated ones which had evidence of blood differentiation (Figure 33).

A disadvantage associated with Con A treatment was that the presence of the lectin caused a certain number of the cells to adhere to the sides and bottom of the culture flask. This, therefore, caused a reduction in the concentration of suspended cells. A consequence of this was that there were usually fewer and smaller aggregates formed in the presence of Con A than in the control. This is a possible reason for the lack of well differentiated aggregated formed in the presence of Con A; there may be a requirement for a critical number of cells to be present for effective differentiation to occur. The presence of 10 mM α-methyl-D-mannoside reduced the cell adhesion to the glass. The aggregates formed in the presence of lectin plus hapten were more similar to the control aggregates in terms of their organization and the extent of cell differentiation. It was difficult to determine if any one cell type was preferentially adhering to the flask, however, the early aggregates formed in the presence of Con A appeared to have the same relative proportions of cell types as the controls. This may be observed by comparing Figures 20 and 21. The flasks were siliconized before each experiment and BSA was included during the first 24 hours of culture in an effort to reduce the lectin-mediated adhesion to the glass substrate. Neither of these methods were particularily effective in this regard.

Of all the lectins tested, only RCA was observed to have a profound effect on aggregation. At concentrations greater than 0.5 μg/ml RCA there was a marked reduction to a complete inhibition of aggregation. This was apparent during the first hour of incubation with 5 μg/ml RCA; at lower concentrations (0.05 to 0.5 μg/ml) the cells initially aggregated but these aggregates usually disintigrated after about 24 hours. Aggregates which were formed in the presence of 0.01 μg/ml and lower concentrations of RCA were cultured for a total of 72 hours and then fixed, sectioned, stained, and examined. A 72 hour control aggregate is illustrated in Figure 35. In this aggregate initial cell sorting had taken place and the

Figures 35 to 39. Aggregates formed from trypsin dissociated blastoderm cells and cultured for 72 hours. Stained with Haematoxylin and Eosin. The bar is 100 µm.

Figure 35. A control aggregate showing cell sorting into loose and compact phases with the latter tending to be internalized. A large cavity is present.

Figure 36. An aggregate which had been treated with 0.001 µg/ml RCA during the first 24 hours of culture. Typical cell sorting patterns are evident.

Figure 37. An aggregate which had been treated with 0.005 µg/ml RCA during the first 24 hours of culture. Note that the compact phase is present external to the loose phase.

Figure 38. An aggregate which had been treated with 0.01 µg/ml RCA during the first 24 hours of culture. Note the random cell associations.

Figure 39. An aggregate which had been treated with 0.1 µg/ml RCA plus 5 mM thiodigalactoside during the first 24 hours of culture. There is evidence of cell sorting into loose and compact phases. The compact phase is present at the surface and internal to the loose phase.

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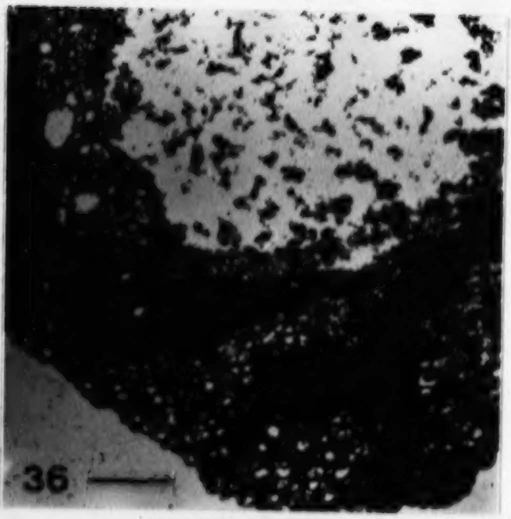
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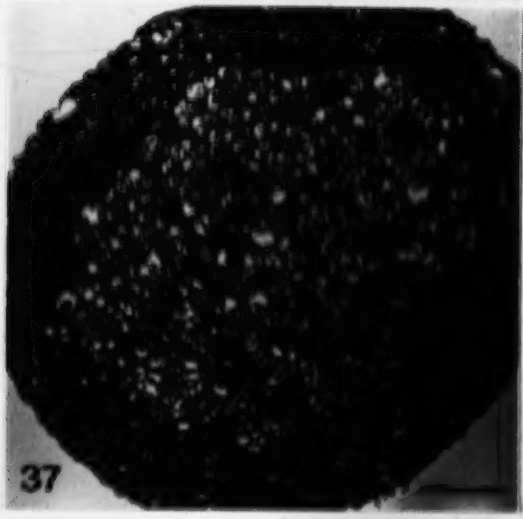
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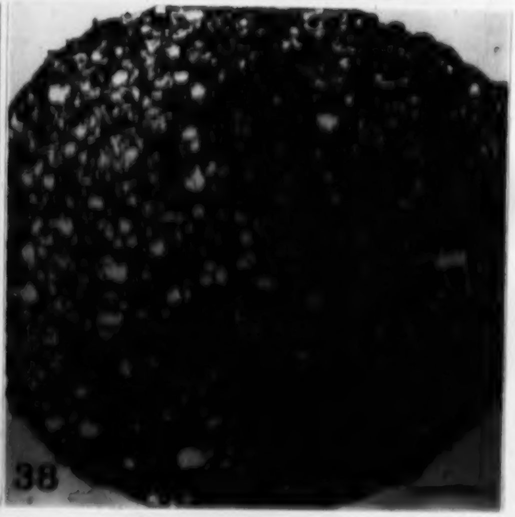
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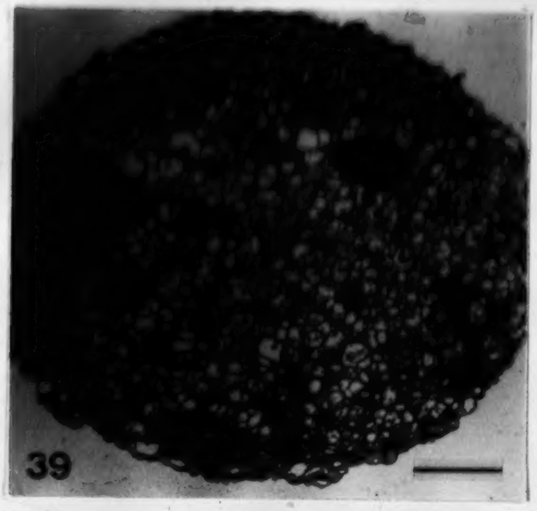
39

external, loosely packed, cells were beginning to take on the appearance of yolk sac endoderm. Within the densely packed cells there were numerous small, irregular cavities. This is apparently a prelude to the formation of the inner trabecular network of cells which was common in the older aggregates. In the aggregate illustrated in Figure 35 there was a large central cavity. The effects of RCA treatment may be observed by comparing the control aggregate in Figure 35 to lectin treated aggregates illustrated in Figures 36, 37, and 38. A RCA concentration of 0.001 μg/ml did not affect the aggregate morphology appreciably (Figure 36). However, the aggregates formed in the presence of 0.005 and 0.010 μg/ml RCA were usually smaller and exhibited altered morphologies from the controls. At 0.005 μg/ml RCA (Figure 37), there was still some evidence of cell sorting, however, the pattern was different from that observed in the controls. In the aggregate illustrated in Figure 37 the compact phase had sorted externally to the loose phase in an apparent reversal to the usual situation. In aggregates formed in 0.010 μg/ml RCA there was no evidence of any cell sorting (Figure 38). The cellular associations appeared to be randomized throughout the aggregate. The hapten TDG was moderately effective at reducing the RCA-mediated inhibition of aggregation and cell sorting. An aggregate formed in the presence of 5 mM TDG and 0.1 μg/ml RCA (a concentration that usually inhibited aggregation) is illustrated in Figure 39. In this case there was evidence that cell sorting had occurred. The aggregate had an appearance similar to aggregates formed in much lower concentrations of RCA. This may be seen by comparing Figure 39 with Figures 36 and 37. β-lactose was also similarily effective at reducing the effect of RCA on aggregation.

DISCUSSION

The quantitative analysis of lectin-mediated agglutination utilized in this study has made it possible to expand upon earlier observations on the agglutinability of cells from gastrulating chick embryos (Zalik and Cook, 1976; Phillips and Zalik, 1978). In addition, this work has provided information on the differential agglutinability of cells from different embryonic regions. The novel features determined from this study are as follows. a) Cells dissociated from the area pellucida, as well as those obtained from the endoderm of the area opaca are agglutinated to the same extent by WGA, RCA, and SBA. b) These two cell populations exhibit different Con A-mediated agglutinabilities; the AP cells being more agglutinable with this lectin. c) The high inherent agglutinability of AOEn cells with WGA, RCA, and Con A is further enhanced by trypsinization; this difference was not detected in previous studies utilizing a visual assessment of agglutination (Zalik and Cook, 1976). d) In the area pellucida, trypsinized cells subjected to neuraminidase treatment are more agglutinable with SBA than EDTA dissociated, neuraminidase treated, cells. e) RCA greatly interferes with the ability of blastoderm cells to form cohesive aggregates and undergo cell sorting. WGA, Con A, and succinylated Con A have a negligible effect on these processes.

The interpretation of lectin-mediated agglutination studies is complicated by the fact that lectin specificities are no longer assumed to be directed against just simple sugar molecules. Initially, lectin specificities were defined on the basis of the monosaccharide which most effectively inhibited an agglutination reaction or the lectin-induced precipitation of a large oligosaccharide. It was assumed that if a lectin caused an agglutination reaction, then the specific monosaccharide molecule was present at the cell surface, most likely as a terminal residue in a glycoprotein or glycolipid. It has become apparent, however, that lectins are not only capable of recognizing oligosaccharides, but also, the types of linkages between sugar residues; moreover, these proteins are also capable of binding to subterminal carbohydrate residues (Nicolson, 1974). The specificity of Con A appears to be primarily directed against α-mannopyranosides and α-glucopryranosides. Although the monosaccharides are effective inhibitors of Con A-mediated agglutination, Goldstein et al. (1973) have reported that Con A is capable of interacting with di- and trisaccharides. These may be present as terminal residues or internally within an oligosaccharide chain (Goldstein et al., 1973; Goldstein, 1976; Narasimhan, 1979). WGA also appears to be capable of interacting with internal residues of oligosaccharides (Goldstein et al., 1975). These authors have shown that the specificity of WGA is complementary to a trisaccharide of β-1-4 linked N-acetyl-D-glucosamines. The trisaccharide was considerably more effective as an inhibitor of WGA-mediated precipitation of carcinoembryonic antigen (a protein-polysaccharide complex) than the di-and monosaccharides of GlcNac. WGA is also capable of interacting with sialic acid. Burger and Goldberg (1967) showed that neuraminidase treatment prevented WGA-mediated agglutination of tumor cells but sialic acid did not act as a hapten for WGA. Bhavanandan and Katlic (1979), however, found that GlcNac compounds, as well as N-acetylneuraminic acid were effective inhibitors of WGA-mediated hemagglutination, whereas, neuraminic acid-β-methyl ketoside and N-glycolylneuraminic acid were not inhibitory to this reaction. This suggests the necessity of the N-acetyl group for the haptenic interaction with WGA. Stanley et al. (1980) have suggested that there are several classes of sialic acid residues present on the surface of Chinese hamster ovary cells; these classes are defined on the basis of their interactions with WGA. Cell mutants lacking different groups of sialic acid residues displayed a differential binding of WGA. Both RCA and SBA appear to interact exclusively with terminal residues on oligosaccharides (Nicolson, 1974; Lis and Sharon, 1977). Cell agglutination mediated by RCA (the 120,000 M.W. species) is most strongly inhibited by β-linked disaccharides with galactose at the nonreducing end. SBA appears to be specific for α- or β-linked N-acetyl-D-galactosaminyl residues (Lis and Sharon, 1977). Lectin receptors on cell surfaces seem to be primarily glycoproteins; these molecules may interact with lectins in a different manner than the monosaccharides used as haptenic inhibitors of lectin-mediated reactions. Allen and Johnson (1976) have presented evidence which suggests that lectins with similar monosaccharide specificities may bind to different cell surface oligosaccharides. Binding studies of labelled lectin to tumor cells and Scatchard plot analysis revealed that broad bean lectin and lentil lectin, both specific for mannose, could bind either to a single receptor or to different receptors with similar affinities. Con A and pea lectin apparently bound to two different receptors. Broad bean and lentil lectins were effective inhibitors of pea lectin binding but Con A was not as effective in this regard. Conversely, pea lectin was a poor inhibitor of Con A binding, suggesting that Con A and pea lectin were binding to different receptors. The binding of all of these lectins was inhibited by methyl-α-D-mannoside. In a similar study, Feller et al. (1979) examined lectin binding to human fibroblasts. They concluded that Con A could bind to all pea and lentil lectin receptors but the latter two lectins only bound to 25% of the Con A binding sites. WGA also interfered with Con A binding; this was concluded to be either a result of the two lectins competing for GlcNac residues or to a steric hindrance resulting from two lectins binding to different residues on the same glycoprotein.

Additional information on the specificites of lectins can only serve to make these molecules more useful as sophisticated probes for cell surface glycoconjugates. Continued research on the nature of lectin specificities may reveal that lectins are capable of recognizing portions of the core lipid or protein molecule of the glycoconjugate. In the present study, however, it is only possible to speculate on the nature of the cell surface lectin receptors on the basis of cell agglutination alone. It is reasonably safe to suggest that the sugars α-D-mannopyranoside, D-galactose, and N-acetyl-D-glucosamine are present within surface glycoproteins of chick blastoderm cells, and that these molecules are capable of participating in an agglutination reaction. This assumption is based on the ability of both AOEn and AP cells to be agglutinated by Con A, RCA, and WGA. The fact that GlcNac was not completely effective as a haptenic inhibitor of WGA-mediated agglutination suggests that this lectin may be interacting with other carbohydrates. As mentioned previously, this may be due to the greater specificity of this lectin for trisaccharides of GlcNac or it could be a result of WGA interacting with sialic acid. Sanders and Anderson (1979) have also suggested that WGA may bind to glucosamine residues of hyaluronic acid in the extracellular matrix of embryonic chick cells. The fact that SBA does not cause an agglutination reaction of the cells unless they are first treated with neuraminidase suggests that N-acetyl-D-galactosamine residues may be present subterminally to neuraminic acid residues. This was suggested by Zalik and Cook (1976), although, they did not rule out the possibility that the presence of neuraminic acid poses a steric hindrance to the binding of SBA and/or prevents a reorientation of SBA receptors into positions favourable for an agglutination reaction.

One aspect of the cell agglutination mediated by Con A and RCA which was not observed in previous studies (Zalik and Cook, 1976; Phillips and Zalik, 1978) was the incorporation of the subcellular particles into the agglutinated cell masses. This response was interesting in that the particles did not agglutinate in the absence of cells. In the presence of cells, this agglutination did not appear to be merely a passive entrapment of particles since this result only occurred in the presence of Con A and RCA and not with the other two lectins. The subcellular particles consisted of extracellular yolk platelets and the products of cell lysis such as intracellular yolk platelets, cell organelles, membrane vesicles and other cell fragments. It was not established whether any one population of these particles was being selectively agglutinated. It is likely that the membrane vesicles and cell fragments possessed surface glycoproteins which could act as agglutinable lectin receptors. Rutishauser and Sachs (1975) examined lectin-mediated agglutination occurring between cells immobilized on nylon fibres and free cells in suspension. They observed that if both the immobilized and free cells were prefixed, the agglutination reaction was inhibited. The separate fixation of either the free or the immobilized cells did not prevent agglutination . This suggested that in an agglutination reaction between two cells, receptor mobility is only required on one of the cells. These authors suggested that this mobility may be required to bring the receptors on one cell into alignment with those on the other cell, thus, enabling a lectin molecule to form a cross link between the two cells. An equivalent situation to that of Rutishauser and Sachs (1975) could explain the RCA and Con A-mediated cell to subcellular particle agglutination observed in the experiments reported here. Even though the subcellular particles possess surface lectin receptors, they may not have the "cytoplasmic machinery" required to redistribute the receptors. An alternate explanation for this phenomenon is suggested by the work of Whitehead and Marcus (1975). These authors have examined the binding of lymphocytes to Con A covalently coupled to Sepharose beads. They observed that cells bound to the beads in multilayers rather than in monolayers as one would expect. The cells at the periphery of the aggregates were presumably not adhering as a result of the Con A as the lectin was only present at the surface of the bead. The hapten α-methyl-D-mannoside inhibited both the adhesion of cells to the Sepharose beads as well as the cell-cell adhesion. It was surmised that the Con A-mediated adhesion of the cells to the beads resulted in altered cell surface properties, perhaps as a result of lectin-induced receptor redistribution, which made the cells more adhesive. This alteration of surface properties could be transferred from cell to cell over several layers. These lectin-induced surface changes may be responsible for the binding of the subcellular particles to the cells observed in the present experiments. Con A and RCA could induce surface alterations causing the cells to become "sticky" to the particles.

Previous studies from this laboratory on adhesion and lectin-mediated agglutination of early embryonic chick cells have all revealed that there is a great deal of variability in the behaviour of blastoderm cells after experimental manipulation. This poses difficulties in the interpretation of experimental results and necessitates large numbers of experimental replicates in order to verify the consistency of a certain response. The variability is possibly due to any of a number of factors such as minor fluctuations in the dissociation procedure, the cell concentration or in the amount of yolk and cellular debris present in the final cell suspension. Umbreit and Roseman (1976) reported that the age of the maternal hen from which an embryo was obtained affected the adhesiveness of liver cells from 12 day embryos. The livers of embryos derived from young hens were more easily dissociated than were the livers of embryos from older hens. The cells of embryos from young hens were also shown to aggregate better than cells of embryos from old hens. The experiments reported in this study were performed over a time period of two years on embryos obtained from several different flocks of hens (the flocks were changed approximately every six months). It subjectively appeared that the embryos from younger hens were more readily dissociated into clean cell suspensions and that these cells exhibited a greater lectin-mediated agglutinability than did cells dissociated from embryos derived from older hens. This effect, however, remains to be established conclusively. Milos et al. (1979b) have examined the levels of endogenous chick lectin in supernatants of dissociated cells. Variations in the level of lectin seem to correlate with the adhesiveness of the cells. Freshly dissociated cells which remain in saline for periods of time appear to release lectin into the suspending medium; these cells exhibit poor adhesiveness. If the cells are washed and resuspended in fresh saline the level of lectin activity in the supernatant is reduced and the adhesive ability of the cells is increased. Their results suggest that the released soluble lectin in the supernatant may interfere with adhesion. Variations in the lectin activity present in a cell suspension may account for the observed variations in the adhesiveness of the cells. It is possible that the presence of endogenous lectin activity may affect exogenous lectin-mediated cell agglutination. Despite the fairly high degree of variation in the experimental results, the extensive repetition of the experiments coupled with the statistical analysis was considered sufficient to take into consideration the degree of variability present in each treatment. The results obtained in this study are considered to reflect valid characteristics of the agglutinability of early embryonic cells.

The dose response of blastoderm cells to Con A treatment is interesting in two aspects: a) a difference in the agglutinability of AOEn and AP cells was revealed; and b) high concentrations of lectin inhibited the agglutinability of both cell populations. The enhanced agglutinability of AP cells over those of AOEn may be due to the presence of a higher number of surface receptors for Con A on AP cells; alternatively it may reflect a cell-type difference in the availability of agglutinable Con A receptors. Lectin binding studies need to be performed to distinguish between these two possibilities. Current thinking seems to favour the idea that differences in lectin-mediated agglutinability may reflect alterations in the mobility and distribution rather than in the number of lectin receptors. This was alluded to in the introduction where the results of numerous investigations on the enhanced agglutinability of protease treated and transformed cells were discussed (see Nicolson, 1976). Another possibile explanation of the enhanced Con A-mediated agglutinability of AP cells is that the lectin may have a higher affinity for particular glycoprotein receptors at the surfaces of these cells. Studies involving analysis by Scatchard plots of lectin binding have demonstrated the presence of binding sites with varying affinities. Allen and Johnson (1976) have shown that murine ascites tumor cells possessed two or more receptors with differing affinities for Con A and pea lectin. Similarily, Feller et al. (1979) provided evidence for high and low affinity Con A binding sites on human fibroblast cells. Stanley and Carver (1977, 1978) examined the binding of labelled WGA to Chinese hamster ovary cells and determined that at least four classes of receptor sites were present, three of which exhibited positive cooperativity in WGA binding. Examination of WGA-resistant mutants revealed that although the total number of binding sites was similar, there were differences in the intermediate affinity sites.

In the previous study of lectin-mediated agglutination of chick blastoderm cells (Phillips and Zalik, 1978) the highest concentration of Con A tested was 20 μg/ml. In this investigation it was found that lower than maximal agglutination occurred at lectin concentrations greater than 25 μg/ml. It is possible that the binding of large numbers of lectin molecules to the cell surface glycoproteins causes a steric hindrance and interferes with the redistribution of receptors to positions favourable for agglutination. Con A has been shown to inhibit the orientation of lymphocyte surface receptors into patches and caps when the lectin is present in doses greater than 5 μg/ml (Edelman et al., 1973). Con A has also been shown to have a slight cytotoxic effect although the mechanism of this toxicity is uncertain (Nicolson, 1974; Lis and Sharon, 1977). It is possible that the inhibition of agglutination observed at high concentrations of lectin may be due to its cytotoxic effect although it should be mentioned that high concentrations of RCA, with a potent cytotoxic effect to cells in culture (Olsnes and Pihl, 1977), did not inhibit agglutination.

The dose responses of both AOEn and AP cells to WGA, RCA, and SBA were more typical in that the graphs of agglutination versus lectin concentration were sigmoidal in appearance. Increases in the lectin concentration above that required for maximal agglutination did not significantly alter the response. It is apparent from the results of the lectin dose experiments that RCA elicits the greatest agglutination response in both AOEn and AP cells. As discussed previously, this may be due to any of a number of factors such as a large number of surface receptors, a high affinity of RCA for the galactose groups of the surface glycoproteins, or perhaps a greater accessibility of this particular lectin receptor. The response is worth noting, since RCA has a similar carbohydrate specificity to the endogenous chick blastoderm lectin described by Cook et al. (1979). Thiodigalactoside and β-lactose are potent inhibitors of agglutination mediated by both RCA and the blastoderm lectin. If the endogenous blastoderm lectin is indeed involved in adhesive events one would expect an abundance of its particular receptors at the cell surface. The results of the RCA-mediated agglutination seem to indicate an availability of a large number of surface receptors for the blastoderm lectin. However, one should bear in mind that, although two lectins may have similar specificities for simple sugars, they may not bind to the same oligosaccharide receptor at the cell surface. With continued research into their system, however, it may prove feasible to utilize RCA as an economical means of isolating cell surface receptors for the endogenous chick lectin.

The results of WGA, RCA, and SBA dose response experiments suggest that cells of the AP and AOEn possess similar numbers of agglutinable receptors for each of the above lectins. It still remains to be established whether the different cell types present in the area pellucida exhibit differential agglutinabilities with any of these lectins. These experiments were not performed due to technical difficulties associated with obtaining the large numbers of dissociated cells required for the quantitative assay. The work of Sanders and Anderson (1979) suggests that cells migrating through the primitive streak they do not contain as many binding sites for WGA as do the cells of the epiblast or the mesenchyme lateral to the primitive streak. Whether these differences could be detected in an agglutination assay is uncertain as the dissociation procedure may very likely alter the distribution and perhaps availability of lectin binding sites.

The sensitivity of the quantitative analysis of lectin-mediated agglutination over a visual assessment was revealed in the experiments on the effect of trypsin treatment on agglutination. Zalik and Cook (1976), utilizing a visual assessment of agglutination, could not detect an enhanced agglutinability after trypsinization of blastoderm cells. In the present investigation, however, trypsinization was shown to enhance the agglutinability of AOEn cells with Con A, WGA, and RCA. This increase, found to be statistically significant, was small and could not be detected reliably with a visual assessment. Zalik and Cook (1976) did note that trypsinization was required to render embryonic liver cells agglutinable with WGA. These authors suggested that trypsinization may remove a masking agent from the WGA receptors, or alternatively, it may induce a change in the distribution of surface receptors into positions more favourable for agglutination. These explanations also may possibly apply to the trypsin-mediated enhancement of agglutination in the blastoderm cells. Rosenblith et al. (1973) showed that Con A binding sites on protease treated cells were more readily orientated into patches than were the binding sites on nontreated cells, suggesting that the protease treatment enhances the mobility of surface receptors. Trypsin has been shown to disrupt the organization of microtubular and microfilaments (Furcht and Wendelschafer-Crabb, 1978; Britch and Allen, 1980). This trypsin-mediated disruption of the cytoskeletal elements may possibly be involved in causing the increased mobility of lectin receptors, which in turn, may be responsible for the enhanced agglutinability of protease treated cells.

The trypsin-mediated enhancement of WGA-mediated agglutinability of AOEn cells was interesting in that it appeared that the control incubation at 37°C reduced the agglutinability of the cells compared to that of the 4°C controls. The trypsin treatment at 37°C merely restored the agglutinability back to the levels in the 4°C control. This trend was also observed with RCA although in this case the differences between the two temperature controls were not significant. With Con A, however, the 4°C and 37°C control cells were agglutinated similarily, while trypsin enhanced the agglutinability over both controls. It is possible that the incubation at 37°C in CMF PCS causes alterations of the lectin receptors such that the cells are less agglutinable. Trypsin treatment may prevent, or actively interfere with this loss of agglutinability, perhaps by enhancing the receptor mobility. These experiments indicate, however, that already at this early developmental stage trypsin sensitive elements may be involved in controlling some lectin receptors and maintaining them in a nonagglutinable condition.

The effect of trypsinization on SBA-mediated agglutination of AOEn cells was not examined as it was felt that the number of experimental manipulations involved was too harsh on the cells. However, in the case of AP cells it was observed that cells which had been dissociated with trypsin and subsequently treated with neuraminidase were more agglutinable with SBA than were EDTA-dissociated, neuraminidase-treated AP cells. The agglutinability of AP cells with Con A, WGA, and RCA was not affected by the dissociation technique. This apparent discrepancy may possibly be explained on the basis of the experimental protocol and incubation temperatures in each of the cases. The EDTA dissociation technique involved incubating the cells on ice, whereas, the trypsin dissociation was performed at 37°C in CMF PCS. When SBA-mediated agglutination was tested, both EDTA and trypsin dissociated AP cells were subjected to an additional incubation with neuraminidase at 37°C in a 1:1 mixture of PCS and CMF PCS. It is possible that incubation at 37°C caused the differential reduction in agglutinability of EDTA dissociated AP cells compared to the cells obtained by trypsinization. This may have been similar to the situation in AOEn control cells which showed a decreased WGA and RCA-mediated agglutinabity when incubated at 37^{o}C.

Sanders and Zalik (1976) reported that cells dissociated from gastrulating chick embryos were capable of reaggregating and undergoing a certain degree of histotypic differentiation. This investigation has substantiated these findings and has provided some additional information on the effect of lectins on adhesion and subsequent aggregate morphogenesis. It was interesting that in the control aggregates and those formed in the presence of WGA, Con A, or succinylated Con A there appeared to be a preponderance of mesodermal structures. With the exception of the external yolk sac-like cells, the majority of the differentiated structures within the aggregate were of apparent mesodermal origin. It is not certain as to whether this was due to an initial selection of cells during the dissociation procedure or as a result of the culture conditions. The fact that similar results were obtained with two different dissociation techniques tends to rule out the former possibility. There are numerous inductive processes occurring during gastrulation and it is to be expected that the dissociation of the blastoderm and subsequent culturing may disrupt some of these processes. The formation of a yolk sac-like morphology by extraembryonic endoderm cells has been shown to be an intrinsic property of these cells and will occur in the absence of any other embryonic tissues (Milos et al., 1979a). The tendency of these cells to assume an external location has been suggested by Eyal-Giladi et al. (1975) to be a response to the localization of the nutrient supply. The other differentiated tissues which were observed in the aggregates, blood elements, tubules, and connective tissue, have been assumed to be derived from mesoderm.

The results of the lectin treatment on aggregation are difficult to interpret as the mechanisms and consequences of lectin binding to cell surfaces are poorly understood. In studies where lectins have been shown to cause disruption of cellular recognition and adhesive phenomena the results have been interpreted to be due to a lectin-induced alteration of the mobility or distribution of cell surface molecules. In many cases, the binding of an exogenous ligand or lectin to a cell surface receptor has been shown to induce a capping of the bound receptor and a subsequent endocytosis of the capped area (Taylor et al., 1971; Aubin et al., 1980). While this response does indicate lectin-induced surface changes it also provides the cell with an effective method for removing the lectin from its surface, thus, providing the possibility of an intracellular site of action of the lectin.

The lack of effect of WGA, Con A, and succinylated Con A in this investigation may have been due to the low lectin concentration compared to the concentrations utilized by other investigators. In this study the lectins were tested at low concentrations in order to minimize the possibility of cytotoxic effects. Higher lectin concentrations would also have caused significant amounts of agglutination which would have interfered with analysis of the results. Evans and Jones (1974) used 200 μg/ml of a nonagglutinating form of Con A to inhibit the aggregation of embryonic chick neural retina and skeletal muscle cells. Steinberg and Gepner (1973), however, reported that 20 μg/ml of a univalent Con A did not block adhesion of neural retina cells. Prinz and Von Figura (1978) demonstrated an inhibition of adhesion of human skin fibroblasts to a plastic surface with Con A concentrations of 3 to 500 μg/ml. A concentration of 10 μg/ml Con A was required to inhibit the adhesion of embryonic retina cell aggregates to cell layers (Letourneau, 1979) and to disrupt the migration of primordial germ cells (Lee et al., 1978). In the above mentioned studies the cytotoxicity of Con A was discounted as being responsible for the observable effects. This has been the subject of a certain degree of controversy, however; Steinberg and Gepner claim that greater than 20 μg/ml univalent Con A is cytotoxic to neural retina cells.

The only lectin which affected aggregation under the experimental conditions of this investigation was RCA. As RCA has a similar specificity to the endogenous blastoderm lectin (Cook et al., 1979) this result may provide some support for the hypothesis that the endogenous lectin and its receptors are involved in cell surface mediated events. Caution is required in the interpretation of the effects of RCA as this lectin has been shown to be quite toxic to cultured cells, apparently by interfering with protein synthesis (Saltvedt, 1976; Olsnes and Pihl, 1977). Vernay et al. (1979) recently reported that RCA inhibited the adhesion of embryonic chick fibroblasts. They surmised that the inhibition was due to a toxic effect, since the presence of this lectin caused an inhibition of incorporation of (^{3}H) leucine and (^{14}C) glucosamine into trypsin sensitive glycoproteins at the cell surface. Fluorescent labelled RCA was observed to be internalized by the fibroblasts within one hour after its addition. The effect of RCA on adhesion of chick blastoderm cells requires additional investigation to determine if the inhibition of cell adhesion and sorting is due either to its interaction with galactose containing surface glycoproteins or to its effect on protein synthesis.

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